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Effect of Blood of Myasthenia Gravis Patients on Acetylcholine Sensitivity of Prostigminized Frog and Leech Muscle.*

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The idea of a circulating toxic factor in the blood of patients with myasthenia gravis as set forth by Bramwell and Campbell,[1] and Oppenheim[5] received confirmation in a clinical experiment by Walker.[7] She reported a case of myasthenia gravis in which, by cutting off the circulation in both arms and maximally exercising the arms, she was able to induce drooping of the eyelids and then generalized weakness by merely releasing the tourniquets. This observation suggested to us the possibility of demonstrating in the blood of patients with myasthenia gravis a factor which would depress the acetylcholine sensitivity of prostigminized leech dorsal and frog rectus muscles. In a search of the literature we could find no such previous attempt.

Methods. The dorsal muscle of the leech and the rectus of the frog were each set up in a test tube and the response to acetylcholine measured according to the method of Chang and Gaddum,[2] with slight modifications. The muscle was immersed in 25 cc of frog Ringer's

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* We wish to express our gratitude to Doctor Louis Leiter for the use of the medical laboratories, and to Doctor Karl Harpuder for his helpful guidance.

[1] Bramwell, H. and Campbell, E., *Brain,* 1900, **23**, 277.

[2] Chang, H. C. and Gaddum, J. H., *J. Physiol.,* 1933, **79**, 255.

[3] Harvey, A. M., and Lilienthal, J. L., Jr., *Bull. Johns Hopkins Hosp.,* 1941, **69**, 566.

[4] McEachern, D., *Medicine,* 1943, **22**, 1.

[5] Oppenheim, H., *die myasthenische Paralyse,* Berlin, S. Karger, 1901.

[6] Torda, C., and Wolff, H. G., *Science,* 1943, **98**, 224.

[7] Walker, M. B., *Proc. Roy. Soc. Med.,* 1938, **31**, 722.

solution, prostigmine added to a concentration of 2.5×10^{-5}, and then acetylcholine in concentration varying from 1×10^{-7} to 2.5×10^{-7} depending on the reactivity of the individual muscle. This was in each case the control contraction, to which we compared the contraction obtained under the same concentrations of prostigmine and acetylcholine in the presence of 1 cc of different blood samples. Nine normal subjects and 3 patients with myasthenia gravis were the blood donors. In all cases the blood was drawn from a deep antecubital vein, with the arm at rest and again after the forearm muscles had been exercised to a state of fatigue with the tourniquet applied just above the antecubital fossa. In 4 experiments the blood was drawn after the tourniquet had been loosened. Blood was allowed to stand for 6 hours at 0°C before being used; it was then centrifuged and the serum used. In all experiments save one the serum was clear; in this experiment the blood was purposely hemolyzed by drawing with a wet syringe, and the serum used contained many hemolyzed red blood cells. The Ringer's solution was always brought to a pH of 7, and air constantly bubbled through the solution. All materials were at room temperature when used.

Results. In the presence of clear serum. Neither the normal serum nor the serum from patients with myasthenia gravis had any effect on either the prostigminized frog or leech muscle before the addition of acetylcholine. The height and the slope of the contraction to acetylcholine did not appreciably differ from that obtained in the control (a) whether there was serum from normal subjects or patients with myasthenia gravis present, (b) whether the serum was obtained before exercise or after exercise, or (c) whether the blood was drawn with the tourniquet applied or with the tourniquet loosened.

In the presence of serum containing hemolyzed red blood cells. Both the height and the slope of contraction obtained in the presence of serum containing hemolyzed red blood cells were greater than the control, but there was no difference between that obtained with normal blood and that with blood from patients with myasthenia gravis.

Discussion. Although the literature has abundant references to the possible presence of a circulating toxic factor in myasthenia gravis, laboratory confirmation has been lacking. By injecting the freshly drawn venous blood of myasthenic patients, McEachern[4] attempted unsuccessfully to demonstrate a "curariform" effect in cats. Other authors such as Harvey and Lilienthal[3] spoke of a curare-like substance in patients with myasthenia gravis. Our work shows that in the six-hour-old sera of myasthenic patients there is no curare-like substance, as this type of blood did not interfere with the acetylcholine sensitivity of prostigminized leech dorsal or frog rectus muscle. Any possibility of demonstrating the presence of this factor in red cells was obscured by the stimulating effects of potassium and lecithin released from hemolyzed red cells. One is not justified, however, in denying the presence of a toxic factor outright, for the following three reasons. First, the methods may have been inadequate to isolate, preserve, and demonstrate this substance. Second, is the fact that we used frog and leech muscle, presumably from healthy animals. There is a difference between the presumably normal frog and leech muscles and the easily-fatigued muscles of a myasthenia gravis patient. Moreover, as Walker showed in her experiment, the muscles of a myasthenia gravis patient are not all affected to the same extent necessarily. These facts suggest that if there is a circulating factor, it can produce its effects only upon a susceptible muscle. Finally our experiment was limited to demonstrating the effect of myasthenia gravis serum upon the action of previously synthesized acetyl choline. It is therefore possible that the circulating factor affects not the action but the synthesis of acetyl choline. Torda and Wolff[6] have recently shown that frog brain incubated in the presence of serum from patients with myasthenia gravis synthesizes less acetylcholine than it does in the presence of serum from normal people or patients with other debilitating diseases. They suggest that the defect in myasthenia gravis is in the synthesis of adequate amounts of acetylcholine to maintain muscular contraction, and that the faulty synthesis may be due to several factors, one of which is the presence of inhibitor substances.

Summary. Attempts were made to experimentally demonstrate a circulating factor in the blood of normal people and of patients with myasthenia gravis before and after exercise to a fatigued state, which would depress the response of prostigminized frog and leech muscle to acetylcholine. Before the addition of acetylcholine to the perfusion fluid it was found that neither normal serum nor serum from myasthenic donors had any effect upon the prostigminized leech or frog muscle. Serum, whether from a normal or myasthenic donor, had no significant effect upon the acetylcholine sensitivity of either frog or leech muscle. Although hemolyzed blood from both normal and myasthenic donors increased the acetylcholine sensitivity of prostigminized leech and frog muscle, there was no quantitative difference in the effect of the two types of hemolyzed blood. A circulating factor depressing the acetylcholine sensitivity of frog and leech muscle could not be demonstrated with our methods in myasthenic patients. Some reasons for the non-detection of this hypothetical factor and some theories as to the etiology of myasthenia gravis were discussed.

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Penicillin: Miscellaneous Pharmacological Results.*

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Penicillin has surprised all investigators in its non-toxicity. Various phases of acute and chronic toxicity, and *in vitro* activity, are reported in this paper. The results corroborate those of van Dyke[1] in the paucity of adverse pharmacodynamic effects.

1. *Effect on Excised Organs.* The contractions of strips of pregnant guinea pig uterus in an excised-organ bath were not affected by 1, 2 or 10 cc of a solution containing 1500 u of penicillin per cc. Similarly, several strips of rabbit abdominal aorta were not influenced by from 1 to 5 cc of the same penicillin, while 1 cc of a 1:5 million dilution of epinephrine produced contraction.

2. *Vomiting in Pigeons.* Two pigeons were given penicillin intravenously, 4000 and 7500 u respectively in 5 cc of water, without the production of vomiting, or other symptoms, in 30 minutes.

3. *Anticatalytic Effect.* Two hundred and fifty individual tests were made of the *in vitro* effect of penicillin on the liberation of oxygen from hydrogen peroxide by a catalyst.[2] The results were similar, irrespective of whether Fuller's earth or collargol served as the catalytic agent. Penicillin from 6 different sources, in high concentrations, invariably inhibited the liberation of oxygen, while in low concentrations (50 units or less in the system), it produced no effect. It was at first hoped that

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Dr. Alvin J. Cox, Jr., of the Department of Pathology assisted in the pathological examinations, and Capt. Arthur L. Lack, A. U. S., in the examination of conjunctival vessels. Part of the penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutics and other agents of the National Research Council, and part was supplied by Dr. Charles Clifton of the Department of Bacteriology, Stanford Medical School.

[1] van Dyke, H. B., PROC. SOC. EXP. BIOL. MED., 1944, **56**, 212.

[2] Hanzlik, P. J., and Cutting, W. C., *Science*, 1943, **98**, 389.

this might be a means for assaying penicillin, but inactivation of the penicillin by incubation at 37° C for 4 days, by boiling, by autoclaving at 15 lb pressure for 20 minutes, and by autoclaving penicillin acidified to pH 2, did not greatly affect the inhibitory power on the production of oxygen. This suggests that the inhibitory substance is not penicillin, although it could conceivably parallel the original penicillin content. A sample experiment is shown in Table I, in which oxygen was available in each tube from 1 cc of 1% hydrogen peroxide plus 1 cc of 0.02% collargol.

TABLE I.
The Inhibition of Catalytic Action by Penicillin and Inactivated Penicillin.

Test agent	Height of gas column produced	Height of gas column produced after autoclaving penicillin
Control	6.0 cm.	....
“	5.6	....
Penicillin, 5000 u.	0	0 cm.
“ 500 u.	0.8	0.3
“ “	0.7	0.4
“ 50 u.	5.6	5.2
“ “	5.3	4.8
“ 5 u.	4.4	5.2
“ “	5.0	5.4
“ 0.5 u.	4.8	5.7
“ “	4.8	5.9

Ten per cent solutions, or saturated solutions, of several agents which were considered to be possible inhibitors of penicillin were added to the system in 0.5 cc quantities, after neutralization. Whereas 6.2 cm of gas were produced in the control tube, all the tubes containing penicillin (500 u) showed complete, or almost complete, inhibition, the greatest quantity of gas produced being only 0.4 cm (superoxol). The agents included sodium benzoate, potassium permanganate, para-aminobenzoic acid, sodium citrate, Solution of Formaldehyde, U.S.P., hydrogen peroxide 30%, sodium salicylate, urea, propylene glycol, acetone, and ferric chloride (unsatisfactory).

Tyrothricin was found not to affect the production of gas in the catalytic system.

4. *Acute Meningeal Irritation.* Because of clinical suggestion that penicillin might be irritating to the meninges, it was injected intracisternally into 2 dogs, one being given 2000 u in 2 cc of solution, and one 200 u in 2 cc of solution 5 times during 6 days. Estimations of the spinal fluid protein were made on the first, second and fifth days, with the following results: Dog 1, receiving 2000 u daily, 62, 41 and 19 mg per 100 cc; Dog 2, receiving 200 u daily, 85, 88 and 187 (bloody) mg per 100 cc; Control Dog 3, given 1 cc physiological salt solution daily, 62, 147 (bloody), 99 and 209 (bloody) mg per 100 cc. From this it was concluded that the greater concentration of penicillin did not produce an increase in spinal fluid protein.

On the 7th day, the dogs were sacrificed. The brain of the dog which had received 200 units of penicillin daily showed slight hemorrhages at the site of the injections and several collections of mononuclear cells in the meninges over the base of the brain. These latter may have been in response to the bleeding. The brain of the dog which had received 2000 units of penicillin daily showed only a very few collections of mononuclear cells, similar to those in the first dog, and was otherwise not abnormal, from which it was concluded that the stronger penicillin solution did not damage the meninges.

5. *Chronic Toxicity.* One rooster was given 500 to 1000 u of penicillin intramuscularly daily except Sundays for 22 days. At autopsy no gross abnormalities were seen. Four rats were each given 500 u of penicillin subcutaneously in 5 cc of physiological salt solution daily except Sundays for 6 months. During this time they appeared healthy and raised several litters. Examination of conjunctival blood vessels under magnification at 2 months showed no abnormalities of blood flow. When sacrificed at the end of the period of injection, pathological sections were made of all the principal organs and tissues. Locally at the site of injection there were numerous old and new hemorrhagic areas, excessive accumulations of fibrous tissue and some foreign body giant cells. The injections were not made with sterile precautions, and the above changes may have been due to trauma and the accidental introduction of microorganisms and foreign particles. Otherwise, the only abnormal pathological changes were a few clumps of lymphocytes in the brain of one of the animals

and an excessive number of eosinophils in the bronchial walls, a finding not uncommon in control rats. The conclusion is that chronic toxicity of penicillin in rats is not demonstrable with the doses used.

6. *Percutaneous Absorption.* Penicillin was applied to the freshly clipped abdominal skin of rabbits by gentle rubbing on two occasions. For the first trial, 1000 units of penicillin were dissolved in 5 cc of monoethylether of diethylene glycol (carbitol) and applied over 15 minutes; for the second, 5000 units were dissolved in 2.5 cc of the same vehicle and applied over 1 hour. In neither case was there any measurable penicillin in the blood at the end of the period of application. A mixture of 4000 units of penicillin in 0.05 gm of zephiran was administered sublingually to a man. No penicillin appeared in the urine during the next 45 minutes.

Summary. In spite of the lethal effect which penicillin exerts on bacteria, power to alter or poison normal physiological processes significantly has not been demonstrated. It has no demonstrable effects on contraction of the isolated aorta or pregnant uterus, or on the vomiting reflex of pigeons; it produces no serious meningeal irritation; and, in small doses, it produces no chronic tissue changes in rats. Although the penicillin used inhibited the liberation of oxygen from hydrogen peroxide by catalysis, the inhibition persisted after inactivation of the penicillin and, therefore, may be due to some other substance in the impure products. Monoethylether of diethylene glycol did not promote its percutaneous absorption.

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Collodion Particle Adsorption of Equine Encephalomyelitis Virus.

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The flocculation of talcum powder or of organisms such as *Proteus vulgaris* or *Eberthella typhi* in *Escherichia coli* filtrate upon the addition or anti-coli serum was reported as early as 1898 by Nicolle.[1] That protein is adsorbed upon various particles has been shown by changes in physical properties such as isoelectric point or cataphoretic mobility.[2-4] The agglutination of collodion particles as a result of the reaction of the adsorbed protein with specific antiserum was observed by Jones[5] and by Freund.[6]

Collodion particle suspensions have been used in many studies involving their adsorption of antigen.[7, 8] In most cases, protein solutions containing relatively large amounts of antigen have been employed for adsorption upon the collodion particles. The adsorption of virus antigens from tissue suspensions, where the specific antigen is in very small amount, upon cells of *Serratia marcescens* with the demonstration of agglutination by virus antisera has been reported by Roberts and Jones[9] and Weil.[10] Later, Pearson[11] was unable to show agglutination with specific antisera and collodion particles or cells of *S. marcescens* which had adsorbed material from

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[1] Nicolle, C., *Ann Inst. Pasteur.*, 1898, **12**, 161.

[2] Coulter, C. B., *J. Gen. Physiol.*, 1921, **3**, 309, 513.

[3] Northrup, J. H., and DeKruif, P. H., *J. Gen. Physiol.*, 1922, **4**, 655.

[4] Loeb, J., *J. Gen. Physiol.*, 1923, **5**, 395.

[5] Jones, F. S., *J. Exp. Med.*, 1927, **46**, 303.

[6] Freund, J., Proc. Soc. Exp. Biol. and Med., 1930, **28**, 65.

[7] Zozaya, J., *J. Exp. Med.*, 1932, **55**, 325.

[8] Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1940, **38**, 365.

[9] Roberts, E. C., and Jones, L. R., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 75; 1942, **49**, 52.

[10] Weil, A. J., *J. Immunol.*, 1942, **45**, 187.

[11] Pearson, H. E., *J. Immunol.*, 1944, **49**, 117.

TABLE I.
Agglutination of W.E.E. "sensitized" collodion particles.

Series	Source of virus for sensitization of collodion particles	Antiserum used*	Agglutination: Serum dilutions†					
			1:2	1:4	1:8	1:16	1:32	Saline
1	Allantoic fluid	W.E.E.	----	+	+	+	—	—
	"	Normal	+	+	—	—	—	—
2	"	W.E.E.	—	—	—	—	—	—
	"	Normal	—	—	—	—	—	—
3	"	W.E.E.	+	+	—	—	—	—
	"	Normal	+	—	—	—	—	—
4	"	W.E.E.	+	—	+	—	—	—
	"	Normal	—	+	—	—	—	—
5	Chick embryo	W.E.E.	—	—	—	—	—	—
	"	Normal	—	—	—	—	—	—
6	Mouse brain	W.E.E.	—	—	—	—	—	—
	"	Normal	—	—	—	—	—	—

* The sera are designated according to the nature of the mouse brain used to immunize the rabbits.

† Agglutination is recorded as positive whether partial or strong. Complete agglutination was not observed.

influenza, poliomyelitis, St. Louis encephalitis or eastern equine encephalomyelitis virus suspensions. We have attempted to adsorb the virus of western equine encephalomyelitis (W.E.E.) upon collodion particles and to cause their agglutination by specific hyperimmune rabbit sera.

Methods. Collodion particle suspensions were prepared according to Cannon and Marshall's[8] modification of Loeb's[12] method. Three sources of virus were used: allantoic fluid of infected 11-day chick embryos; clear supernate from suspensions of 10% infected chick embryo in 0.8% saline; and clear supernate from 1% infected mouse brain suspension in 0.8% saline containing 1% rabbit serum. These tissues were harvested under conditions in which the virus was close to maximal titer. No attempt was made to control pH during adsorption; buffers were avoided since they may cause the precipitation of collodion particle suspensions.[8] The collodion particles were added to the virus suspensions in amounts varying from one to two volumes per volume of virus suspension, and of a turbidity which when diluted twice would be comparable to tube 3 or 4 of McFarland's standards.[13] After standing in the ice-box overnight, or sometimes 36 hours, the collodion particles were washed 3 times in 0.8% saline. They were then diluted to a turbidity equal to tube 5 of McFarland's standards and 0.5 ml introduced into 0.5 ml of each of a series of double dilutions of anti-W.E.E. or of control antiserum.

Hyperimmune sera were prepared by repeatedly injecting rabbits with mouse brain suspensions of a mouse-adapted strain of W.E.E.* and control antisera were prepared by similarly inoculating rabbits with suspensions of normal mouse brain. We used the allantoic fluid from W.E.E. infected chick embryos because the low protein[14] and high virus content[15] of this tissue might be expected to reduce non-specific reactions to a minimum.

Attempts to demonstrate agglutination with virus antigen-antibody systems. Collodion particles which had been treated with virus-containing allantoic fluid gave agglutination with about the same dilutions both of W.E.E. and of control antisera (see Table I).

Infected chick embryo suspensions and mouse brain suspensions were then used as virus sources. 11-day-old chick embryos moribund with W.E.E. were triturated in 0.8 % saline to give a 10% suspension, and the clear supernate obtained after centrifuging for one hour at 4000 rpm in an angle head, was used. The mouse brain suspension was pre-

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[12] Loeb, J., *J. Gen. Physiol.*, 1922, **5**, 109.

[13] McFarland, J., *J.A.M.A.*, 1907, **49**, 1176.

* Obtained originally from Dr. Schlessinger at the Rockefeller Institute.

[14] Needham, J., *Chemical Embryology.* The Macmillan Co., New York, 1931.

[15] Bang, F. B., *J. Exp. Med.*, 1943, **77**, 337.

TABLE II.
Amount of virus adsorbed by collodion particles.

Dilution of materials	W.E.E.C.E.S.[*] adsorbed with collodion particles	W.E.E.C.E.S. control
10^{-4}	2/2[†]	2/2
10^{-5}	2/2	2/2
10^{-6}	0/2	1/2
10^{-7}	0/2	0/2

[*] Western equine encephalomyelitis chick embryo suspension.

[†] Numerator = number of 10-day chick embryos infected.

Denominator = number of chick embryos inoculated.

pared as is the complement fixing antigen of Casals and Palacios.[16] The brains of mice moribund with W.E.E. were triturated to give a 10% suspension in 0.8% saline containing one percent rabbit serum; after freezing and thawing 5 times and centrifuging as with the chick embryo suspension, the clear supernate was employed.

After adsorption from the chick embryo suspension or the mouse brain suspension, the collodion particles gave no agglutination with either the W.E.E. hyperimmune serum or with the control antisera (see Table I).

Tests upon antisera. The hyperimmune sera were tested for neutralizing antibody by introducing virus dilutions into undiluted serum, and about 10^4 mouse protective units per ml were observed.

Complement fixation tests with the Casals and Palacios[16] mouse brain antigen and the antisera inactivated for half an hour at 65° C to remove non-specific antibody to brain protein showed that these W.E.E. antisera fixed complement at dilutions of the order of 1:40; the normal mouse brain antisera gave incomplete fixation at dilutions of 1:8 or less.

Amount of virus adsorbed by collodion particles. Since the agglutination reactions were essentially negative although the sera contained antibody to W.E.E., we wondered whether sufficient virus had been adsorbed on the collodion particles to give a positive reaction. Titration of virus suspensions before and after adsorption with collodion particles indicated that the virus was only slightly adsorbed.

TABLE III.
Effect of decreasing percentage of ovalbumin in 2% protein solution during sensitization of collodion particles. Overnight exposure.

% of protein as ovalbumin	Agglutination: anti-ovalbumin serum dilutions 1:20	1:40	1:80	1:160	1:320	Saline
100	+	+	+	±	—	—
66	+	+	±	—	—	—
40	+	+	+	—	—	—
20	+	±	±	—	—	—
6	—	—	—	—	—	—
2.5	—	—	—	—	—	—

+	complete agglutination
±	partial agglutination
—	no agglutination

The following experiment is typical: Fifteen ml of supernate of a 25% W.E.E. chick embryo suspension in 0.8% saline were added to 7½ ml of collodion particle suspension of a turbidity which when diluted 4 times matched tube 3 of McFarland's standards. This mixture was allowed to stand overnight in the ice box and a portion was then removed and centrifuged 20 minutes at 4000 rpm. The supernatant fluid was treated about half an hour with 1:20,000 Zephiran (a mixture of high molecular alkyl-dimethyl-benzyl-ammonium chlorides)[17] to kill the bacteria introduced with the collodion particles and tenfold dilutions were made in tryptose broth for titration with 10-day chick eggs. A control portion similarly treated but not adsorbed was also titrated. The results of these titrations are recorded in Table II.

In such determinations, the decrease of virus in the suspension treated with collodion particles was little more than that observed in the control suspension. Therefore, the washed collodion particle suspensions when diluted to two or three times the original volume for the agglutination test would contain less than 10 chick embryo infective units per ml. Merrill[18] has accumulated data indicating that for a virus of this size about 10^{11} virus particles per ml would be necessary for a visible immunological reaction. The sensitivity of collodion particle agglutination is not likely to surmount such a differential.

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[16] Casals, J., and Palacios, R., *Science*, 1941, **93**, 162.

[17] The Personnel of U. S. Naval Laboratory Research Unit No. 1, *Science*, 1942, **96**, 543.

[18] Merrill, M. H., *J. Immunol.*, 1936, **30**, 169.

TABLE IV.

Effect of decreasing percentage of ovalbumin in 2% protein solution during sensitization of collodion particles. One-half hour exposure.

% of protein as ovalbumin	Agglutination with anti-ovalbumin serum dilutions									
	1:2	1:4	1:8	1:16	1:20	1:40	1:80	1:160	1:320	Saline
100	....	....	....	....	+	+	+	+	—	—
75	....	....	+	+	±	±	—	—	—	—
50	....	±	—	—	—	—	—	—	—	—
25	—	—	—	—	—	—	—	—	—	—

+	complete agglutination
±	partial agglutination
—	no agglutination
....	not examined

Effect of the presence of other proteins in agglutination by adsorption. The effect of the large amount of non-specific tissue protein in suspensions of virus-containing tissue was approached artificially. In 2 experiments, when collodion particles were sensitized in mixtures of ovalbumin and sheep serum, the agglutination titer of an anti-ovalbumin rabbit serum decreased with the amount of ovalbumin in the protein mixture. In the first experiment the collodion particles remained in the protein solution overnight, while in the second the exposure was for half an hour. Results of these experiments are given in Tables III and IV.

There is 0.5% protein by micro-Kjeldahl analysis in the supernate from a 10% W.E.E. mouse brain suspension prepared according to Casals and Palacios[16] method for complement-fixing antigen. 0.02 ml of a 10^{-6} dilution of this suspension was infectious for young mice. Assuming 50 virus particles per mouse infectious dose—special methods are necessary to show one virus-particle infectious[19, 20]—and a mass of 3.7×10^{-15} g per particle, then each ml of suspension would contain about 10^{-6} g of virus. The virus content would then be 0.0001% and the portion of protein as virus would be 0.02%. The decrease in agglutinating titer of an antiserum with the percentage decrease of specific protein in a mixture of proteins indicates that the low percentage of virus in respect to tissue might be responsible for negative results.

Conclusions. Only a small amount of the virus of western equine encephalomyelitis is adsorbed upon collodion particles from the clear supernates of virus-containing tissue suspensions during an overnight period in the icebox. This amount is not sufficient to give a positive agglutination reaction with specific antiserum. Experiments with collodion particles sensitized with protein mixtures indicate that it will probably be necessary to concentrate and partially purify the W.E.E. virus in order to obtain collodion particles adequately sensitized for agglutination with virus antisera.

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[19] Parker, R. F., and Rivers, T. M., *J. Exp. Med.*, 1936, **64**, 439.

[20] Parker, R. F., *J. Exp. Med.*, 1938, **67**, 725.

14891P

Vaccination of Mice Against Typhus.

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Ever since the discovery of its susceptibility by Nicolle *et al.*[1] the guinea pig has been the animal most widely used in typhus studies. Its reaction to established murine strains,

TABLE I.
Vaccination of Mice with Epidemic Typhus Vaccine.

							Results			
							After 24 hrs.		After 7 days	
Exp. No.	No. of mice used	Vaccine	Vaccine dose	Yolk sac infecting dose	Strain	Route*	Living	Dead	Living	Dead
1	10 test	30% yolk sac	0.5 cc	1.5 MLD	Epidemic	i.v.	10	0	....	....
	10 controls		0				0	10	....	....
2	10 test	20% " "	0.5 "				10	0	....	....
	10 "	10% " "	0.5 "	<1 "	"	"	10	0	....	....
	20 controls		0				5†	15	....	....
3	8 test	10% " "	0.5 "				8	0	....	....
	8 "	10% " "	0.25 "	3 "	"	"	8	0	....	....
	16 controls		0				0	16	....	....
4	8 test	10% " "	0.1 "	3 "	"	"	7	1‡	....	....
	8 controls		0				0	8	....	....
5	16 test	10% " "	0.5 "	<1 "	Murine	i.p.	16	0	8	8
	16 controls		0				13	3	4	12

* i.v.—intravenous; i.p.—intraperitoneal.
† Control mice in this experiment slightly heavier than test mice.
‡ Mouse in poor condition at time of challenge inoculation.

when a scrotal reaction occurs regularly, is quite satisfactory. With the epidemic disease, however, where a definite pattern of febrile reaction is the only evidence to be relied on for interpretation, the guinea pig leaves much to be desired. Wohlrab[2] reported that white mice could be infected with murine typhus by various routes, though he did not obtain one hundred percent morbidity. Various workers have shown that murine typhus could be carried in passage in the mouse, but that the epidemic strain was lost after a few transfers.[3,4] By means of intraperitoneal inoculation with murine infected yolk sacs of embryonated eggs, Gildermeister & Haagen[5] were able to kill mice after 24 or 48 hours. This finding was extended to the epidemic strain by Topping and Bengtson[6] who used the intravenous route of inoculation.

In the course of studies on the development of agglutinins in various species of animals[7] it was observed that mice acquired good titers of this antibody rapidly as the result of inoculation, both with vaccine and with infectious material. It was felt, therefore, that they would be useful as test animals for typhus vaccines, the early formation of agglutinins suggesting that they could be tested for immunity much sooner than has been possible in the case of guinea pigs. This proved to be the case as will be shown below.

Materials and Methods. 30% ether-extracted formalin inactivated yolk sac vaccines were prepared from Breinl epidemic and Wilmington murine strains of typhus; 10% material was made from this. All vaccinations were performed by the intraperitoneal route, employing a single dose of vaccine. Test mice and controls (C.F.W. strain of Swiss mice from Carworth Farms) about 4 weeks of age weighing 11 to 14 g were selected at the same time. All mice were kept under identical conditions at a temperature of 68 to 70°F. The challenge dose was given 7 days after vaccination, and consisted of intravenous or intraperitoneal administration of 0.5 cc of infectious yolk sac suspension. (This material was kept frozen at —70°C in small samples after titration. The highest dilution required to kill 8 out of 8 mice was called 1 mld.) Mice receiving epidemic material were observed for 24 hours; those infected with murine typhus for one week.

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[7] To be published.

TABLE II.
Vaccination of Mice with Murine Typhus Vaccine.

							Results			
							After 24 hrs.		After 7 days	
Exp. No.	No. of mice used	Vaccine	Vaccine dose	Yolk sac infecting dose	Strain	Route*	Living	Dead	Living	Dead
1	8 test	10% yolk sac	0.5 cc	3 MLD	Murine	i.v.	8	0	....	....
	8 controls		0				0	8	....	....
2	8 test	10% " "	0.5 "	>2 "	"	i.p.	8	0	8	0
	8 controls		0				3	5	0	8
3	8 test	10% " "	0.5 "	3 "	Epidemic	i.v.	3	5	....	....
	8 controls		0				0	8	....	....

* i.v.—intravenous; i.p.—intraperitoneal.

Results. Table I. summarizes the results obtained in mice vaccinated with epidemic vaccine, and tested with epidemic and murine typhus yolk sac suspensions.

The results obtained in the first 4 experiments show that it is possible to protect mice with as little as 0.1 cc of 10% yolk sac vaccine against a very large dose of epidemic typhus given intravenously. In order to show that the animals acquire not merely an ability to neutralize the toxic factor in the challenge material but a complete immunity to the whole antigen, further experiments with murine typhus were performed. Mice vaccinated with epidemic vaccine were challenged by intraperitoneal inoculation with infectious murine yolk sac (Table I., Experiment No. 5) and the results compared with those obtained when mice vaccinated with murine vaccine were tested with the homologous strain by the same route (Table II., Experiment No. 2). The amount of epidemic strain vaccine used was insufficient to protect all mice against death from the murine infection, but no early deaths occurred as in the controls, indicating that all the vaccinated animals were protected from the toxic factor in the inoculum and suggesting that this agent is probably identical in both types of typhus.

From Table II it may be seen that the administration of murine vaccine to mice will protect them not only against a very heavy intravenous injection with the homologous strain but against intraperitoneal inoculation as well. Cross-protection against the epidemic strain is only partial however, as shown previously in mice vaccinated with the epidemic vaccine and challenged with the murine strain. Presumably a greater quantity of vaccine in either case would afford complete cross-protection. Further experiments along these lines are in progress.

Summary. A simple and rapid method for the testing of murine and epidemic typhus vaccines in mice is described. By this means, the use of guinea pigs is eliminated and the period of testing shortened by about two weeks as compared with methods currently in use for testing typhus vaccines. The results suggest that the toxic factor in the two strains is identical.

The inoculations were performed by Miss Jane Burrows, to whom we express our thanks.

14892

Electrode Efficiency and Subject Positioning in Electromyography.

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The increasing use of motor unit action currents, in clinical as well as fundamental studies, has led to the need for a better understanding of certain basic technical problems which are constantly encountered. Some of these are: the efficiency of various electrode contacts, the effect of attitudinal reflexes and positioning of the part under examination and the voltage recorded *in vivo*. This report deals with these problems, some of the data having been accumulated from previous work and some from recent experiments.

Methods. Electrode Comparison. Four kinds of electrodes were tested to determine their relative efficiencies: (1) a perforated, circular, silver skin-pad with an outside and inside diameter of 7 mm and 4 mm respectively, (2) an enameled electrode[†] consisting of a 25-gauge hypo needle enameled to within 2 mm of its tip, (3) the core and (4) the shaft of a concentric electrode (copper core in a 22-gauge hypo needle).

The 4 electrodes were arranged in a cluster with the concentric electrode inserted through the aperture of the skin-pad to a depth of ⅝″ to 1″ and the enameled electrode at an angle from outside the skin-pad, its tip being a few mm from the tip of the concentric. An intradermal hypo needle (the indifferent electrode) was placed several cm from the others. Small intradermal wheals of 1% procaine hydrochloride allowed painless insertion of the needles.

A keyboard permitted pairing of the 4 active electrodes in any combination or with the indifferent electrode, giving 10 combinations. These led to dual channel (Offner type 140) balanced amplifiers feeding an audio amplifier, a cathode-ray oscilloscope and Westinghouse bifilar galvanometers for recording on bromide paper. The subject contracted the muscle very slightly until a single unit was heard from one of the electrodes paired with the indifferent. This unit was fed into one channel while the other 9 combinations were fed alternately into the second channel. Since synchronization of different units does not occur in normal muscle, the voltage of any unit recorded on the combinations comprising the test channel, and in step with the unit on the test channel, could be determined.

The muscles used were the deltoid, biceps and triceps brachii, sacrospinalis mass and those of the dorsum of the hand between the thumb and index finger. The subject was seated comfortably at a table with the part being examined in a neutral position, or prone with the head in the midline. Usually the level of contraction employed did not cause visible movement.

Results. Electrode Comparison. A comparison of the pickup efficiency of 4 different types of electrodes using 25 different units revealed that the highest voltage was recorded on an enameled electrode 17 times, on the core of a concentric 7 times, and was identical on the enameled electrode and the shaft of the concentric once. A skin-pad failed to show the highest voltage in any instance. The highest 4 voltages for an enameled electrode were 450, 420, 400 and 300 μ v, for a core 260, 240, 200 and 200, for a shaft 90, 80, 70 and 60, and for a pad 60, 30, 20 and 20. Voltages were picked up by an enameled electrode in 23 of the 25 units, by a core in 20 (plus one questionable), by a shaft in 17, and by a skin-pad in only 8 (plus one questionable).

With 21 of the 25 units there were 2 or more electrodes in the pickup range. In 16 of these the voltage recorded by a single electrode paired with an indifferent was as high or higher than that recorded by pairing two electrodes in the field.[‡] These included instances where the pickup of the enameled electrode, paired with the indifferent, was 160 μ v greater than the combination of this electrode with another in the field (enameled electrode and core) and where the pickup of the core paired with the indifferent was 110 μ v greater than that of the core paired with the shaft. In only 3 units did a pairing of two electrodes in the field record greater voltage than did each single electrode when paired with an indifferent.

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[†] This type of electrode was suggested to us several years ago by Dr. A. S. Gilson.

In no instance did a pairing of two electrodes pick up the unit action potential when each, paired with an indifferent, was out of the field.

With one electrode in the field and two or more showing no pickup (hence serving as additional indifferents), in 10 instances the active electrode recorded more voltage when paired with one indifferent than with another.

Voltage characteristics. Since with *in vivo* preparations electrode position relative to units cannot be duplicated in repeated experiments, there would be no point in attempting to determine the average voltage of a single unit action potential. However, in measuring several hundred unit action potentials under conditions similar to those already described, one of us (J.S.D.) working with Dr. Charles C. Hassett[1] found those higher than 500 μ v to be extremely rare. The highest observed was 1000 μ v, and in this experiment another electrode a few mm away failed to record this action potential.

Pickup range. It was found that although two closely placed electrodes might not show action potentials when paired, a pairing of one of the two with one of a similar distant pair will show action potentials from muscle masses other than those in which the electrodes are placed. In such instances the potentials recorded are those of many units.

Attitudinal reflexes and tension. In addition to the presence, at times, of muscle action potentials from attitudinal reflexes, it was observed that positions of almost imperceptible "tension" of the part being examined resulted in minor contraction. Under normal circumstances this can usually be quieted, at least as far as single unit potentials are concerned, by a more comfortable positioning of the subject.

Discussion. Electrodes with more contact surface than the core of a concentric but less than the bare shaft are most effective in recording muscle action potentials. This is in line with previous observations made with enameled electrodes.[1] With 2 electrodes in the field, cancellation of potentials is apparently greater than augmentation and indicates that a single exploring electrode (with an indifferent) will sample an area more effectively than will 2 electrodes. Cancellation or leakage, or both, make bare needles inefficient for studies of single units, and skin resistance or distance from the active locus, or both, similarly decrease the effectiveness of skin-pads.

The observation of action potentials recorded by one electrode but not by another even though they be separated by only a few mm indicates that care must be exercised before reaching the conclusion that no contraction is present in the area being sampled by only one or two electrodes. While we agree with others[3–5] that normal resting striated muscle has no contraction and hence no action potentials, such an observation can be made only with multiple and efficient electrode pickup. However, since extremely minor voluntary or reflex contraction fires many motor units, the use of the less efficient bare needles or skin-pads is frequently satisfactory for determining relative degrees of contraction in the muscles being observed.

It is apparent that the voltage of a unit action potential as it is recorded with extracellular electrodes at some distance from the active locus is but a fraction of that recorded with intra- and extra-cellular microelectrodes in single fibers.[2]

When resting muscle is being sampled for contraction, the electrodes must be kept close together (within a few cm) to eliminate the possibility of picking up potentials from distant muscles. In addition, attitudinal and myotatic or stretch reflexes from improper positioning must be avoided when resting muscle is being sampled for contraction. This often requires that the subject recline with the face in the midline and with the knees and arms slightly flexed, in a position of absolute comfort.

[‡] In comparing the pickup of various electrodes and combinations, differences of less than 20 μ v were discarded since such differences might result from slight electrode movement.

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On the basis of these observations, it is evident that reports dealing with electromyograms should describe the type and placement of electrodes and the positioning of the subject.

14893

Effects of Morphine, Nembutal, Ether, and Eserine on Two-Neuron and Multineuron Reflexes in the Cat.

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In the spinal cat, small doses of morphine (5 mg per kg) cause diminution in the amplitude of the flexor reflex, but leave stretch reflexes unaffected or slightly enhanced.[1] This differential action has now been investigated by a study of effects on transmission through two-neuron and multineuron reflex arcs in the spinal cat, since it is known that stretch reflexes traverse the former,[2] while reflexes of cutaneous origin traverse the latter[3] pathway. The effects of morphine have been compared with those of nembutal (sodium ethyl, 1-methyl-butyl barbiturate), ether, and eserine (physostigmine salicylate).

Methods. The experiments were performed on adult cats that had been made spinal at the first cervical segment under ether anesthesia. Respiration was maintained artificially and full recovery from the anesthesia was attained before making the study. The lumbosacral spinal cord was exposed by laminectomy and monophasic leads were taken with chlorided silver electrodes from ventral roots L_7 and S_1 severed from their peripheral connections. The nerves to the gastrocnemius muscle, the sural, and one or both branches of the common peroneal nerve were exposed in the ipsilateral hindleg, crushed distally and placed on chlorided silver wire electrodes for stimulation. All exposed nerve tissue was covered with paraffin oil at a temperature of 38°C. Heat was applied to the animal externally. In some experiments, stimulating electrodes were placed on ipsilateral dorsal roots; in others, reflex responses were recorded from a peripheral nerve. Stimulation was effected by single condenser shocks supramaximal for A fibers. Action potentials were recorded photographically by means of an amplifier and cathode ray oscillograph. Ether was administered through the artificial respiration airline by means of a bottle with a regulating valve. The other drugs were freshly prepared (aqueous solutions) and injected intravenously.

Results. A. *Morphine.* Small doses (5 mg per kg) of morphine sulfate regularly enhanced reflex discharges through two-neuron arcs and depressed those through multineuron arcs (Fig. 1). These effects appeared in from 2-15 minutes and persisted for several hours. Larger doses of morphine (15 mg per kg) depressed also the two-neuron arc discharges in experiments in which the reflex was from peripheral nerve to ventral root. The most note-

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The author wishes to thank Dr. D. P. C. Lloyd and Dr. H. S. Gasser for many helpful suggestions and for their interest in these experiments.

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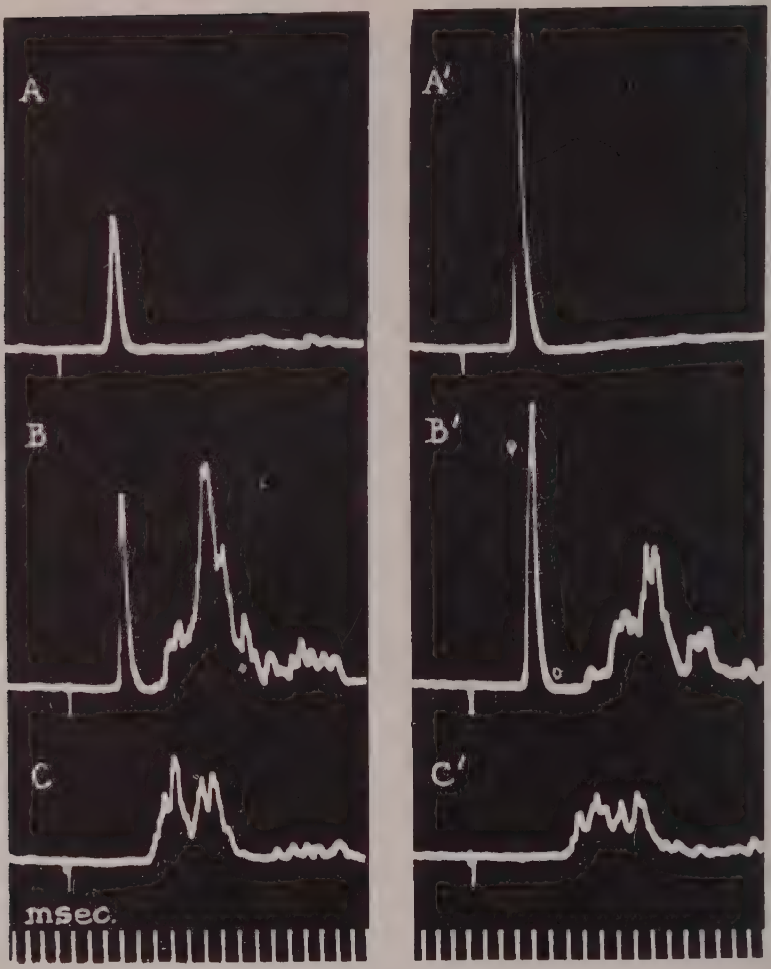

FIG. 1.
Effects of morphine (5 mg per kg i v) on reflex discharges into ventral root L_7 in spinal cat, on stimulation of peripheral nerves by single condenser shocks. A. Two-neuron arc reflex from gastrocnemius nerve. B. Two-neuron and multineuron arc reflex from deep peroneal nerve. C. Multineuron arc reflex from sural nerve. A′, B′, C′, corresponding reflex discharges 24 minutes after injection of morphine. Note enhancement of two-neuron and depression of multineuron reflex arc discharges.

worthy effect of the large doses, however, was on the multineuron arcs. Following the initial depression of the reflexes over these arcs there regularly followed, after 1.5-2 hours, an enhancement of the discharge.

B. *Nembutal.* Following the injection of nembutal, depression of reflex discharges over both two-neuron and multineuron arcs was observed, regardless of dose. Previous injection of eserine or morphine plus eserine did not alter the relationship of the depression with respect to the two forms of reflex discharges.

C. *Ether.* During induction of ether anesthesia, both two-neuron and multineuron reflex arc discharges were progressively de-

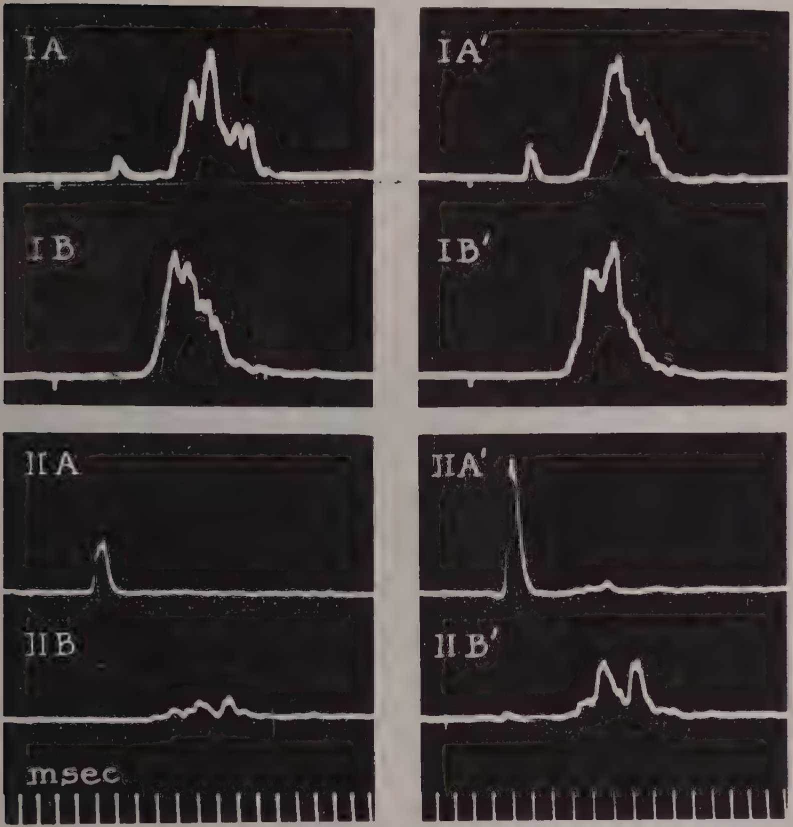

FIG. 2.

I. Eserine as the first agent. A. Two-neuron and multineuron reflexes from deep peroneal nerve. B. Multineuron arc reflex from sural nerve. A′, B′, corresponding reflex arc discharges 22 minutes after injection of eserine (0.25 mg per kg).

II. Eserine, after morphine (5 mg per kg) had enhanced the two-neuron reflex and further depressed the multineuron reflex in a spinal cat in which both reflexes had previously been depressed by nembutal (0.24 mg per kg). A. Two-neuron reflex from gastrocnemius nerve. B. Multineuron reflex from sural nerve. A′, B′, corresponding reflex discharges 7 minutes after injection of eserine (0.25 mg per kg). Note enhancement of both two-neuron and multineuron reflex arc discharges.

pressed, the latter somewhat more rapidly than the former.

D. *Eserine.* Following the injection of eserine (0.25 mg per kg), two-neuron arc reflex discharges were regularly enhanced, while the multineuron arc discharges were little affected. If, however, eserine was injected after morphine, or nembutal plus morphine, both two-neuron and multineuron arc discharges were enhanced simultaneously. (Fig. 2.).

Discussion. Although morphine, ether, and nembutal alike have the power, in the cat, to depress spinal reflexes traversing multineuron arcs, gradation of dose serves clearly to differentiate morphine from the other two agents. Large doses of morphine augment the discharges over multineuron arcs, while large doses of ether and nembutal produce further depression. Additional differentiation appears in the action on two-neuron arc discharges. Morphine, in small doses that depress the multineuron arc discharges, regularly increases the two-neuron arc discharge. An increase of this sort was not observed after administration of any dose of ether or nembutal. Eserine, on the other hand, does enhance two-neuron arc discharges. This is in accord with the excitant action of eserine on the knee jerk reported by Schweitzer and Wright.[4] If the two-neuron arc discharges have previously been enhanced by morphine, they are still further enhanced by a subsequent injection of eserine. Multineuron arc discharges in previously untreated preparations are little affected by eserine. If, however, eserine is injected after the multineuron arc discharges have been depressed by morphine, enhancement of these discharges occurs—an effect similar to that exerted by large doses of morphine.

Summary. 1. In the spinal cat, small doses of morphine (5 mg per kg) enhanced two-neuron arc reflex discharges and depressed those traversing multineuron arcs. After the injection of larger doses (15 mg per kg), depression of multineuron arc discharges was followed by enhancement of these discharges.

2. Nembutal depressed both two-neuron and multineuron arc discharges. These effects were unaltered by previous injection of eserine or morphine plus eserine, and increased progressively with the dose of nembutal.

3. Ether depressed both two-neuron and multineuron reflex arc discharges.

4. Eserine enhanced two-neuron arc discharges, but had little effect on multineuron arc discharges. If, however, morphine had been injected previously, resulting in enhancement of two-neuron and depression of multineuron arc discharges, eserine caused enhancement of both types of reflex arc discharges.

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14894

Hypertensin Destruction by Oxidized Cytochrome.

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The level of hypertensin in blood plasma depends on the relative velocity of its production and destruction reactions.[1]

I. Renin + Hypertensinogen → Hypertensin
(Anaerobic reaction)
/(Protease) → Amino Acids
I. Hypertensin + Enzyme (Anaerobic reaction)
\(Oxidase) → X
(Aerobic reaction)

Under certain conditions, the first reaction is inhibited by some substances found in Vitamin A concentrates,[2] and by some quinones, as methylnaphthoquinone that can stop it completely.[3,4]

So far, the second reaction has been considered to depend on an anaerobic proteolysis only. In fact, most of the organs, mainly the intestinal mucosa, the kidney and the liver destroy hypertensin at different speeds. *In vitro* it has been found that hypertensin is destroyed by renal and intestinal proteinase,[5] by an amino polypeptidase found in renal extracts[6] and in blood plasma,[7] by tyrosinase[8] and by carboxypolypeptidase.[9]

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TABLE I.
Average reduction rate of renal filtration in 13 toads' kidneys.

Hypertensin	60.5%
Hypertensin + reduced cytochrome	70.8%
Hypertensin + oxidized cytochrome	0.

The correlation between the start of the hypertensin activity on the organism and the ischemic and anoxemic phenomena in the kidney, led us to consider that respiration played an important part in the destruction of hypertensin. We investigated this effect, using oxidized cytochrome prepared according to Keilin and Hartree[10] on a Loewen-Trendelenburg[11, 12] preparation, and in a perfused toad's kidney using Plaza de los Reyes technique.[13] To measure the hypertensin activity, the rate of renal filtrate from this preparation was used as a test in a Ringer's circuit without calcium.

Table I. shows the average reduction of this renal filtrate rate in 13 tests. This reduction proves that oxidized cytochrome inhibits the effects of the hypertensive vaso constrictor properties of hypertensin in the kidneys of toads.

TABLE II.

Time of Reduction of oxidized cytochrome, in presence of:	
Renal tissue	24 hrs.
Hypertensin	24 "
Renal tissue + hypertensin	15 min.

Oxidized cytochrome cannot be reduced by renal tissue or hypertensin separately, but, when renal tissue and hypertensin are present together cytochrome is reduced as shown in Table II.

This reduction of cytochrome in the presence of renal tissue and hypertensin is parallel with the disappearance of the vaso constrictor properties of hypertensin (Fig. 1).

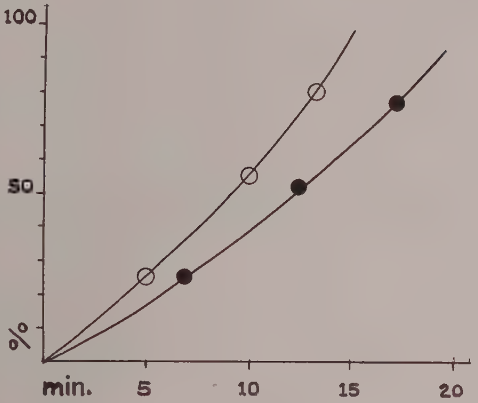

FIG. 1.
Shows parallelism of reduction of cytochrome and inactivation of hypertensin.
○ Reduction of cytochrome.
● Inactivation of hypertensin in the presence of cytochrome.
Ordinates in Figs. 1, 2, 3 and 4 represent percentage reduction in hypertensin.

The action of oxidized cytochrome on destruction of hypertensin by renal tissue was also investigated in the cat. The destruction of hypertensin by anaerobic proteolysis is usually a rather slow process and takes about an hour's time, unless unusually high amounts of enzyme are used. When oxidized cytochrome is added to renal tissue, a rapid de-

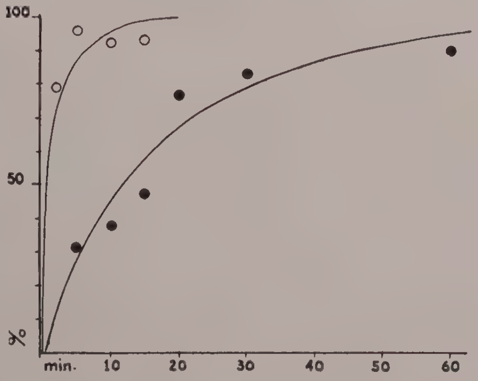

FIG. 2.
Shows that when oxidized cytochrome is added, hypertensin is destroyed much faster.
● Control, *i.e.*, destruction of hypertensin without oxidized cytochrome.
○ Destruction when oxidized cytochrome is added.

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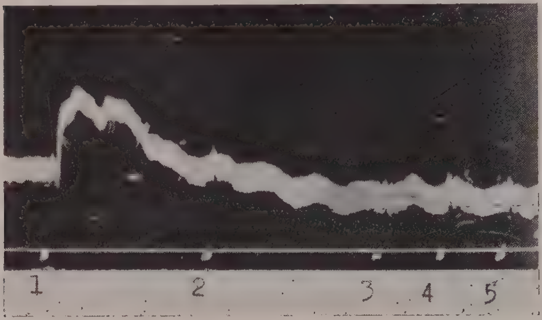

FIG. 3.
This figure represents a typical experiment in which 0.1 cc hypertensin and 0.2 cc renal extract, incubated for different lengths of time, were injected as follows:

No.	Incubation time in seconds	Elevation of carotid arterial pressure mm
1	2	17
2	2	3
3	5	1
4	10	3
5	15	2

At No. 1 no cytochrome was added and the pressure was sharply increased. At 2, 3, 4 and 5 cytochrome was added, and no such increase occurred.

struction of the pressor hormone occurred in a few minutes when tested in the cat. Fig. 2 and 3 graphically show these results.

When 1 cc of N/100 of cysteine or of ascorbic acid was added to the hypertensin renal extract and cytochrome, the hypertensin destruction was sharply retarded as seen in Fig. 4. It is evident that cysteine and ascorbic acid retarded considerably the destruction of hypertensin in both cases *i. e.* with or without cytochrome.

In these experiments renin was prepared according to Swingle, Taylor, Collings and Hays.[14] The hypertensin was obtained by the method of Braun Menendez, Fasciolo, Leloir and Munoz,[15] as modified by Cruz-Coke, Gonzalez, Marish and Hulsen.[16]

It may be of interest to note that the oxygen consumed by the kidney cortex of hypertensive dogs was less than normal,[17] but normal in rats.[18]

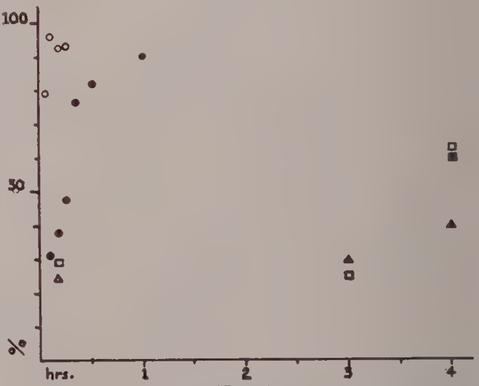

FIG. 4.
This figure shows the destruction of hypertensin when each of the following was added to renal extract:

● renal extract alone (control)
○ cytochrome
■ ascorbic acid
□ " " and cytochrome
▲ cysteine
△ " and cytochrome

Conclusions. The destruction of hypertensin by renal extracts (hypertensinases) was tested by two methods: (1) the rate of filtration in the toad's kidney and, (2) the rise of arterial pressure in the cat.

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In both cases this destruction of hypertensin was markedly accelerated by oxidized cytochrome and sharply retarded by reducing agents such as ascorbic acid and cysteine.

14895

Pityrosporum ovale—A Lipophilic Fungus. Thiamin and Oxaloacetic Acid as Growth Factors.*

Rhoda W. Benham. (Introduced by J. G. Hopkins.)

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The cultivation of *Pityrosporum ovale* by the addition of fats to wort agar has been reported previously[1] and corroborated by Emmons.[2] Of a number of fats and fatty acids, oleic acid seemed the most favorable. In subsequent experiments[3] it was found that if oleic acid was supplied, this organism would sometimes grow feebly on an inorganic solution, similar to Currie's medium, containing NH_4NO_3, KH_2PO_4, $MgSO_4$ and dextrose. Substitution of asparagin as a nitrogen source caused increased growth. If thiamin was added, some growth was regularly obtained even with NH_4Cl as a nitrogen source, and there was increased yield with asparagin. It seemed that further study might clarify the growth requirements of this fungus and throw some light on the function of thiamin in its metabolism.

As a basal media a modified Currie's solution was used. It contained KH_2PO_4, 1 g $MgSO_4$, 0.25 g per liter. Either asparagin 1.25 g or NH_4Cl 2.5 g was added, and a parallel series of flasks containing in addition 50 g of dextrose was usually prepared. A solution of the soluble constituents was distributed in amounts of 50 cc in Erlenmeyer flasks and autoclaved. One-half cc of oleic acid was then added to each flask. Brom cresol green was used as an indicator. Solutions of the supplements to be tested were added by pipette in the following amounts: B_1 5 gamma, ethyl oxalacetate 5 cc of a 5% solution, L-Keto-glutaric acid 2 cc of an M/10 solution. The pH of these media before the addition of oleic acid and the supplements varied from 4.4 to 4.8. The pH was not changed with B_1, but rose to 5 with the ethyl oxalacetate and the keto-glutaric acid. The growth did not seem to be influenced by the pH within the range tested.

Stock cultures were maintained on wort agar tubes to which oleic acid had been added, as previously described. The growth appears, first as tiny cream-colored colonies which later fuse, becoming dry and wrinkled with the color varying from cream to tan or orange. This growth was removed, ground with distilled water to make a heavy suspension. The suspension was then centrifuged, washed several times with distilled water and diluted for use. (0.1 cc of packed fungus cells to 4.9 cc of water). Two or 3 drops of this suspension were used as inoculum. Growth was permitted for 4-6 weeks at room temperature. At this time the growth was harvested and the dry weight obtained. This was done by centrifuging the mold specimen with an equal volume of 95% ethyl alcohol. The sediment was then washed with 50% alcohol and finally

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* The writer wishes to acknowledge her indebtedness to Professor Edgar A. Miller for advice and guidance in conducting these experiments.

[1] Benham, R. W., *J. Invest. Dermat.* 1939, **2**, 187.

[2] Emmons, C. W., *Public Health Rep.*, 1940, **55**, 1306.

[3] Benham, R. W., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 176.

TABLE I.

Showing Average Yields, Maximum and Minimum in Mg Dry Weight.

	+ Dextrose				0 Dextrose			
Media*	No. of measurements	Avg. S.E.†	Max.	Min.	No. of measurements	Avg. S.E.	Max.	Min.
Asparagin	15	44±11.3	123	0	8	24± 8.4	54	0
" + B_1	15	123±14.6	220	52	7	57± 8.8	107	22
" + Oxaloacetic	9	174±17.1	275	110	7	96± 9.6	127	50
Asparagin + Oxaloacetic + B_1	4	114± 2.9	123	110	3	72± 6	85	60
Ammonium Chloride	12	6± 1.6	13	0	8	4± 1.5	10	0
" " + B_1	8	20± 3	40	10	4	24± 6.2	42	10
" " + Oxaloacetic	7	145±20.6	240	80	4	108±30.4	200	45
Ammonium Chloride + Oxaloacetic + B_1	3	171± 4.6	182	165	3	104±26.1	140	40

* The modified Currie's solution with the additions as listed.

† $S.E._{Avg}$ $\sigma/\sqrt{N}$ when σ is the standard deviation of the measurements composing the avearge and measures their variability, and N is the number of measurements.

with acetone. The solid material of the fungus was then dried in a desiccator over calcium chloride and weighed. The results are shown in the accompanying table.

It will be observed that growth in the ammonium chloride media is always less than that with asparagin. The addition of thiamin regularly permitted growth in any of the basal media. When added with asparagin the growth was accelerated and the yield greater than that which occurred in any control tube. When NH_4Cl replaced asparagin, growth with thiamin appeared later and the yield was far less. The addition of dextrose regularly increased growth in the asparagin series, and with thiamin gave yields practically equal to the best obtained. When dextrose was added to the NH_4Cl series, with or without thiamin, there was no increase as compared with parallel flasks without dextrose.

The addition of ethyl oxalacetate caused a striking acceleration of growth. With asparagin the yields were slightly higher than those with asparagin plus thiamin. With NH_4Cl the yields averaged about as good as those with asparagin. The addition of dextrose increased the yield both with asparagin and NH_4Cl. The addition of both thiamin and oxalacetate gave us greater average yield than did oxalacetate alone. The one exception to this would appear to be in the series with NH_4Cl plus dextrose where the addition of both thiamin and oxalacetate gave an average dry weight slightly higher than that with oxalacetate alone. This difference however has been shown to have no significance statistically.†

In two series (results not shown on the table) L. keto-glutaric acid replaced the ethyl oxalacetate with similar results, growth approaching maximum. Lithium pyruvate, on the other hand did not duplicate the effect of the four carbon acids, in fact no growth occurred in any of the flasks to which it was added.

Growth was somewhat irregular in the control tubes in the asparagin series. Usually the yield was small but occasionally approached that obtained by the addition of thiamin. This was thought to be due to some accidental factor and occurred less often when the inoculum was small and carefully washed. This suggested that traces of thiamin may sometimes be transferred with the inoculum. To exclude the possibility that thiamin was present in the asparagin, the material was recrystallized and tested against *Phycomyces blakesleeanus*,[4] and no evidence of thiamin found.

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† When comparing 2 averages, the difference in the averages is compared with the S.E. of the difference where $S.E._{diff.} = \sqrt{S.E.^2_{av._1} + S.E.^2_{av._2}}$. The ratio of difference/$S.E._{diff.}$ must attain a value at least 2.5 to be called clearly significant. Values between 2 and 2.5 are of doubtful significance, and values less than 2 are called non-significant.

Summary and Conclusions. Thiamin when added to a basal medium containing KH_2PO_4, $MgSO_4$ and with asparagin or NH_4Cl causes an increase in the growth of *Pityrosporum ovale*. With asparagin the increase is much greater than with NH_4Cl. However, if ethyl oxalacetate replaces the thiamin, growth in the NH_4Cl medium is just about equal to that in the asparagin medium. The addition of thiamin to the medium containing oxalacetate does not cause any further increase in growth. It would seem then that NH_4Cl and ethyl oxalacetate can be substituted for asparagin and thiamin in the cultivation of *Pityrosporum ovale*. It appears that if a suitable keto acid is supplied neither thiamin nor an amino acid is necessary. On the other hand, the asparagin may furnish both the carbon and nitrogen source needed by the organism, yielding ammonia and a carbon skeleton, probably oxalacetate. Thiamin increases growth when added to the asparagin by hastening the production of the carbon skeleton in some way. When this carbon skeleton is supplied as oxalacetate, thiamin is no longer necessary.

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[4] Robbins, W. J., and Kavanogh, Frederick, *Proc. Nat. Acad. Sci.*, 1937, **23**, 499.

14896

Protective Tests in the Developing Chick Embryo With *Hemophilus influenzae* Type b.

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The assay of the protective power of immune sera versus *H. influenzae* type b is of some practical importance. Serum protection of the white mouse against infection with *H. influenzae* by the mucin method has been demonstrated.[1] However, this method seems not to have given very satisfactory results in routine use, and standardization of therapeutic anti-*H. influenzae* type b serum has been made by the determination of antibody nitrogen.[2] The identity of the precipitating antibody with the mouse protective power in the serum has been ascertained.[3]

The experience with the assay of the protective value of immune sera against *E. typhosa* and *Shigella*[4, 5] suggested an investigation whether a similar test could be developed for anti-*H. influenzae* type B serum.

The developing chick embryo has been previously found to be susceptible to infection with *H. influenzae*,[5,6] and the 6- to 7-day-old chick embryo has been employed as a test animal for the demonstration of the protective effect of sulfathiazole and sulfadiazine.[7] No reports have been found in the literature on the protective effect of immune sera against experimental infection of the developing chick embryo with *H. influenzae*.

Young cultures (5 to 6 h 37°C) either in a digest broth similar to the medium described by Silverthorne and Cameron[8] (but without agar) or in Levinthal broth were used through-

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[1] Fothergill, L. D., Dingle, J. H., and Chandler, C. A., *J. Exp. Med.*, 1937, **65**, 721.

[2] Alexander, H. E., and Heidelberger, M., *J. Exp. Med.*, 1940, **71**, 1.

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[5] Weil, A. J., and McFarlane, J., *J. Immunol.*, 1944, **48**, 291.

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[7] Altura-Werber, E., *J. Bact.*, 1943, **45**, 195.

[8] Silverthorne, N., and Cameron, C., *J. Pediat.*, 1942, **20**, 9.

TABLE I.

Protection by Immune Serum. Ten-day incubated chick embryos received upon the chorioallantoic membrane a mixture of 0.1 ml culture dilution of *H. influenzae* type b (strain No. 62) diluted as indicated plus 0.1 ml serum in 5-fold decreasing dilutions. Ten embryos at each test level. Readings 4 days after infection.

Immune serum	Culture dilution	Microorganisms* (× 1000)	Serum dilutions at 50% endpoint of survival† ml	LD$_{50}$ Virulence control microorganisms
No. 18R37, horse, refined	10^{-3}	48	0.004	2
	10^{-4}	4.8	0.0018	2
O, rabbit	10^{-3}	57	0.0083	4
	10^{-4}	5.7	0.0027	4
N, rabbit	10^{-2}	500	>0.01	2
	10^{-3}	50	0.001	2

* Calculated from plate count of 10^{-6} culture dilution.
† Calculated according to [9].

out the experiments. (Cultures incubated 18 hr do not loose virulence to an appreciable degree.) Dilutions were made in physiological saline solution buffered with M/50 phosphate to a pH of 7.2 to 7.4. We ascertained, in preliminary tests, that the viability of cultures remained unchanged in this fluid even in high dilutions for at least 2 hours. (The majority of bacteria will be killed within one hour if dilutions are made in distilled water.)

Infection can be effected by depositing bacteria on the chorioallantoic membrane and also into the amnion or into the yolk sac. Ten-day-incubated chick embryos proved to be maximally susceptible, the LD$_{50}$ being between 1 and 10 micro-organisms. The use of younger embryos seems to be less advantageous from the point of view of their ability of using antibody to best advantage.

Most chick embryos die within 48 hours after infection. Thus, observation for four days gives an ample margin of safety. Bacteria could be recovered from the heart blood and the chorioallantoic membrane of the infected animals. Heart cultures of serum-protected animals were regularly sterile, while complete disappearance of viable bacteria from the chorioallantoic membrane was not always obtained.

Except for cutaneous bleedings, no gross pathological changes were observed in the embryo (see in this respect also[6]).

Sensitivity to infection decreases rapidly with the growth of the embryo, and the newly hatched chick is not affected by intraperitoneal infection with undiluted culture, that is, an infective dose in the order of 10^9 micro-organisms. In this respect, our experience follows that previously recorded for *E. typhosa.*[4]

As a rule, we used a strain No. 62, which had been obtained in 1939 from Dr. Dingle. A strain Rah, which was kindly sent to us by Dr. Alexander, proved to be equally virulent.

For the test of the protective value of immune serum, infection of the chorioallantoic membrane was routinely used. Immune serum injected into the yolk sac or amnion afforded protection, which was, however, restricted to infective doses not larger than 10^2 micro-organisms, even if serum was introduced 6 to 20 hours previous to infection.

In contrast to this, serum protection was found to be highly effective if the serum was dropped upon the chorioallantoic membrane, and it made no appreciable difference when the serum was applied anytime between 6 hr before and 4 hr after infection. After this time, no significant protective effect was observed. We found that it made no appreciable difference whether the serum was given separately or in admixture with the micro-organisms. Thus, simultaneous introduction of micro-organisms and serums in 0.2 ml total volumes was used in most of our tests, and the following data refer to this method of application.

Tests were made either by assaying a constant volume of serum against decimally decreasing dilutions of culture or—more often—by evaluating the effect of 4- or 5-fold decreasing volumes of serum versus one or 2 dilutions of culture. Constancy of virulence was ascertained in each test by infecting control eggs with culture alone in decimal dilutions from 10^{-6} to 10^{-9}. A series of not-infected eggs were kept as viability controls; this is an important precautionary measure to insure the tests against interference by unforeseen damaging influences. 50% endpoints of survival were calculated according to the method of Reed and Muench.[9]

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[9] Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

The chick embryos are rather uniformly susceptible to infection, but protection is subject to considerable individual variation. Thus, it was found to be advisable to use 8 or, better, 10 chick embryos at each test level. Normal horse and rabbit sera and, similarly, antipneumococcic horse and rabbit sera were found to be devoid of any appreciable protective value in volumes of 0.02 ml.

The immune sera employed in these tests consisted in the main of pooled sera from rabbits that had been treated with increasing volumes of *H. influenzae* type b suspension. Tests were also made with a refined and concentrated horse immune serum 18R37, that had been found by Dr. Heidelberger[10] to contain 1.25 mg agglutinin and 1.15 mg precipitin N/ml.

By way of exemplification, we give in Table I. the results of some of our experiments in which immune sera in 5-fold decreasing dilutions were tested against 2 dilutions of culture. It will be seen that small volumes of immune serum afford protection against infections in the order of 10^3 and 10^4 lethal doses. This latter level of infection is about the upper limit of effectiveness of immune protection. As can be seen from line 5 of the table, infection with well above 10^5 microorganisms is evidently so overwhelming that immune protection is of no avail anymore. This experience is paralleled by that with *Enterobacteriaceae.* [4, 5]

A similar method can also be used for chemotherapeutic experimentation; for instance, the LD_{50} of chick embryos treated by a single injection of 5 mg of sulfadiazine into the amnion immediately before infection with *H. influenzae* was found to be 120,000 microorganisms, the LD_{50} of the controls in this experiment being 2 microorganisms.

Summary. Ten-day-old chick embryos are maximally susceptible to infection with *H. influenzae* type b, the average LD_{50} being between one and ten microorganisms.

Protection against many thousand lethal doses can be obtained by immune serum.

A similar technic can also be employed for chemotherapeutic testing as exemplified by tests with sulfadiazine.

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[10] Personal communication.

14897P

Properties of the Etiologic Agent of Infectious Hepatitis.*

W. P. Havens, Jr. (Introduced by F. G. Blake.)

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Although *infectious hepatitis* has been transmitted experimentally to human volunteers by feeding[1] and inoculating parenterally[2-5] serum which is bacteriologically sterile, the exact nature of the icterogenic agent is yet to be defined. It is believed to be a virus.

During the past few years, numerous reports have appeared, describing the occurrence of jaundice in man following the administration of human blood products and indicating that the icterogenic agent (or agents) of such

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homologous serum jaundice[6-9] and post-vaccinal jaundice[10-15] are filtrable through a Seitz filter and can withstand heating to 56°-60°C for 30-60 min. More recently, certain planned experiments[1, 16, 17] on the transmission of these diseases to human volunteers have corroborated these facts.

The present report describes certain properties of the icterogenic agent of the *naturally* occurring disease, *infectious hepatitis.*

Serum employed in this experiment was obtained during the first 5 days of the experimental disease (pre-icteric phase), which had been induced in 2 human volunteers by feeding fecal material obtained from naturally occurring cases.[†] As such it constitutes *first human passage* material. The serum (designated here as serum M.K.) was stored at dry ice box temperature for 2 months.

For the experiment this serum M.K. was thawed at room temperature, pooled, and filtered through a Chamberlain No. 2 filter, which was demonstrated to withhold a 24-hr broth culture of *Sal. cholera suis.* Immediately afterwards, the filtered serum was heated at 56°C for 30 minutes and stored in the ice box overnight. Cultures of the serum before and after filtration and heating were sterile.

The heated filtrate of serum M.K. was administered in amounts of 1 cc to each of 8 men. These men, who were residents in a penal institution, had volunteered for this experiment. During the experimental period of observation, they were isolated from their fellows. Four men received heated filtrate of serum M.K. orally (and intranasally) and 4 were inoculated sub- and intracutaneously. These men have been observed 125 days. Three of the 4 men who *ingested* the heated serum filtrate contracted moderately severe infectious hepatitis with marked constitutional reaction and jaundice after 23, 24, and 34 days repectively. One of the 4 men who were *inoculated* parenterally with the heated serum filtrate contracted moderately severe hepatitis with marked constitutional reaction and jaundice after 31 days. There had been no hepatitis in the institution for the preceding year, and no other cases have occurred during the period of the experiment.

Summary. An experiment on the transmission of the naturally occurring disease, *infectious hepatitis,* to human volunteers has demonstrated certain properties of the etiologic agent. As in homologous serum jaundice and post-vaccinal jaundice the icterogenic agent of infectious hepatitis is filtrable and withstands heating to 56°C for 30 minutes. The icterogenic agent of infectious hepatitis can be transmitted in serial passage in human volunteers.

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[†] These naturally occurring cases were in American and British troops serving in the Middle East.

14898

Tyrosinase and Phenols. Action of Diversely Activated Tyrosinase on Monohydric and O-Dihydric Phenols.*

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Since the separation and naming of the enzyme, tyrosinase, by Bertrand[1] much confusion has arisen as to its fundamental nature and mode of action. At the present day, it is customary to assume that this enzyme possesses the ability to catalyze the aerobic oxidation of both monohydric and o-dihydric phenols. The ratio of the two activities, however, seems to have become a question of some importance to those especially interested in the isolation and purification of the enzyme. That the two activities belong to the same enzyme complex seems reasonably well established as the result of the work of Nelson *et al.*[2] An inactive form of tyrosinase, called protyrosinase has been found in the diapause egg of the grasshopper, *Melanoplus differentialis.*[3] This has been shown to be converted into the active enzyme, tyrosinase, by a variety of chemical and physico-chemical reagents.[4] Tyrosinase, thus produced, shows similar catalytic characteristics but at the same time exhibits rather different physical properties such as resistance to temperature,[5] degree of molecular fragmentation, etc.[6]

The present communication presents data to show the extent to which variously activated samples of tyrosinase from the egg of the grasshopper are able to catalyze the aerobic oxidation of both monohydric (tyramine-HCl, p-cresol) and o-dihydric phenols (catechol).

Material and Method. Protyrosinase was obtained from the diapause egg of the grasshopper, *Melanoplus differentialis,* as previously indicated.[7] Different lots were standardized uniformly by activation with the anionic detergent, sodium di-octyl sulfosuccinate (aerosol OT), so as to catalyze the uptake of 100 mm^3 of oxygen by 0.3 ml of 0.4% tyramine-HCl at 25°C in 10 minutes. This is referred to as 100% activation of the protyrosinase.[8] Oxygen determinations were carried out in a Warburg manometer as already noted.[8] Various activators were employed in producing the tyrosinase added to the different substrates, *e. g.*, aerosol OT, sodium dodecyl sulfate, $HgCl_2$, heat (70°C-10′), and urea. All activators were used with each lot of protyrosinase and at the same time so that possible differences due to the age of the enzyme sample would be at a minimum. Substrates, made up and used in 0.4% solution or in equivalent concentrations, included tyramine-HCl, p-cresol, and catechol. Total volumes of enzyme, buffer, activator and substrate in manometer cups equalled 3 ml. Comparisons in activity were also made with the relative volumes of reagents as employed by Adams and Nelson.[9] Mixtures with and without the addition of gelatin were also used.[9] Three complete and separate lots of data have been recorded for comparison.

Results. Protyrosinase, when activated by the reagents above mentioned, produces a tyrosinase which, in general, shows marked activity for both monohydric (tyramine-HCl, p-cresol) and o-dihydric phenols (catechol). Such an enzyme, according to the nomencla-

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* Aided by grant from the Rockefeller Foundation for research in cellular physiology.

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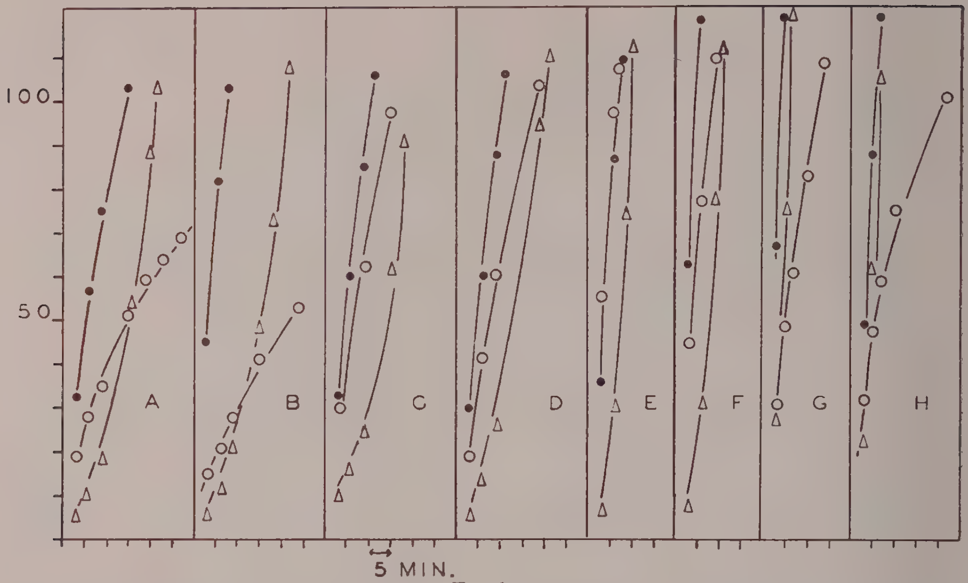

FIG. 1.

Shows action of aerosol OT activated tyrosinase samples upon substrates, tyramine-HCl, p-cresol and catechol. Abscissa, time in minutes; ordinate, O_2 consumption in mm³. Solid dots = tyramine-HCl. Open circles = catechol. Open triangles = p-cresol. A, B, and C are 3 different lots of enzyme similarly prepared. D, same as C but with gelatin added. E, F, G, and H are results obtained by Adams and Nelson method. E = enzyme + aerosol OT + gelatin + substrate. H = enzyme + aerosol OT + gelatin + substrate. E and F for same sample of enzyme. G and H for same sample of enzyme. For further description, see text.

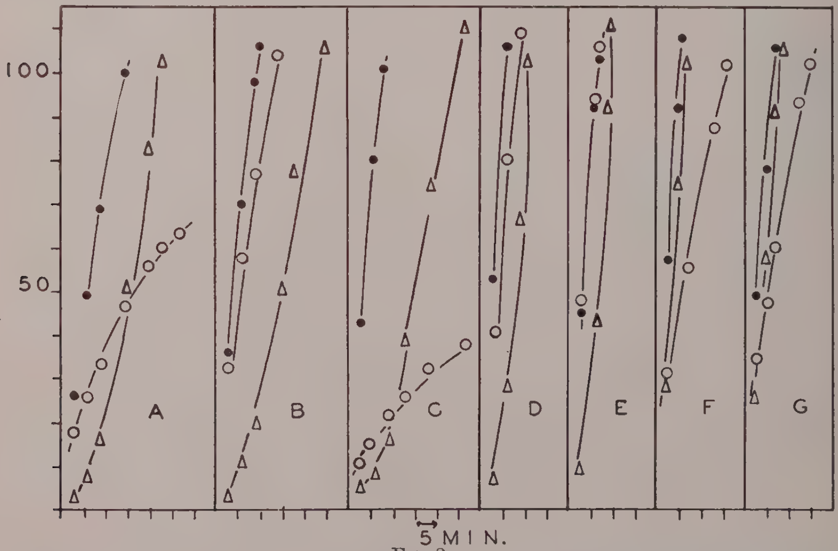

FIG. 2.

Same as Fig. 1. except for sodium dodecyl sulfate. A, B, and C are 3 different lots of enzyme. D, E, F, and G are results obtained by Adams and Nelson method. D = enzyme + NaDS + substrate. E = enzyme + NaDS + gelatin + substrate. F = enzyme + NaDS + substrate. G = enzyme + NaDS + gelatin + substrate.

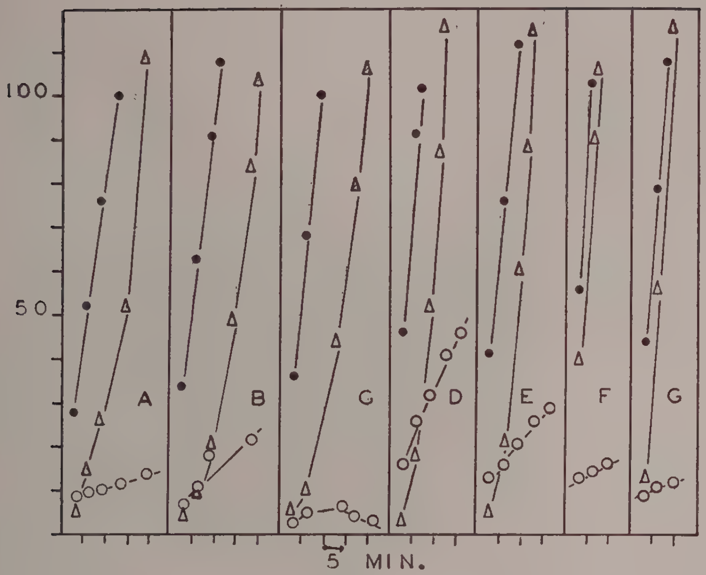

FIG. 3.

Same as Fig. 1 except for $HgCl_2$ ($3. \times 10^{-5}M$). A, B, and C are 3 different lots of enzyme. D, E, F, and G are results obtained by Adams and Nelson method. D = enzyme + $HgCl_2$ + substrate. E = enzyme + $HgCl_2$ + gelatin + substrate. F = enzyme + $HgCl_2$ + substrate. G = enzyme + $HgCl_2$ + gelatin + substrate.

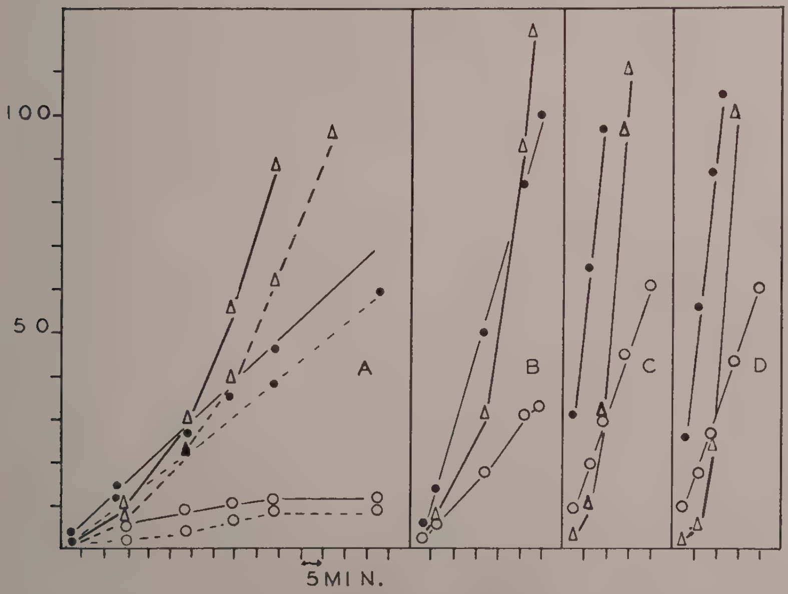

FIG. 4.

Same as Fig. 1 except for urea. Solid lines for 3. M urea; broken lines for 4. M urea. A and B are for different lots of enzyme. C and D are results obtained by Adams and Nelson method. C = enzyme + urea + substrate. D = enzyme + urea + gelatin + substrate.

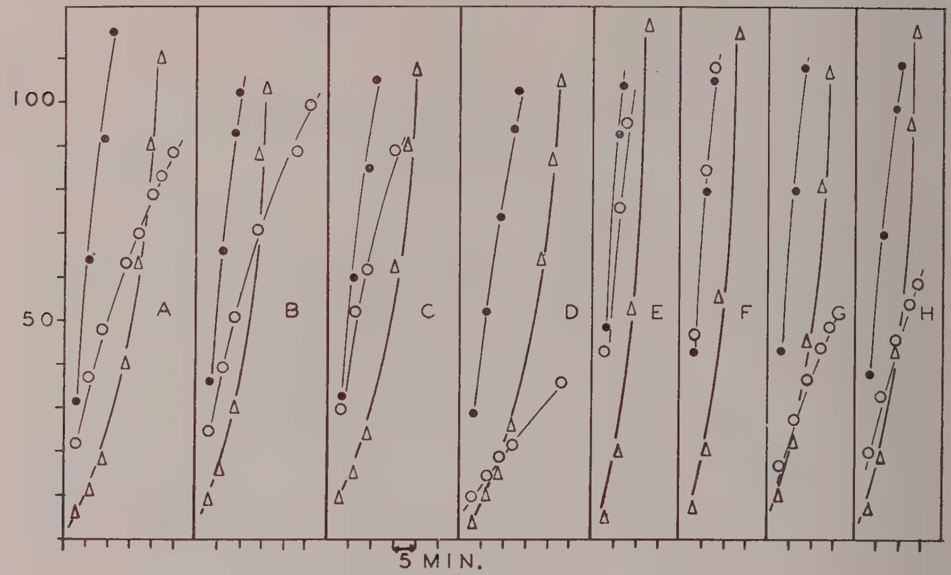

FIG. 5.

Same as Fig. 1 except for exposure to 70°C for 10 minutes. A, B, C, and D are for different lots of enzyme. E, F, G, and H are results obtained by Adams and Nelson method. E = enzyme + heat + substrate. F = enzyme + heat + gelatin + substrate. G = enzyme + heat + substrate. H = enzyme + heat + gelatin + substrate.

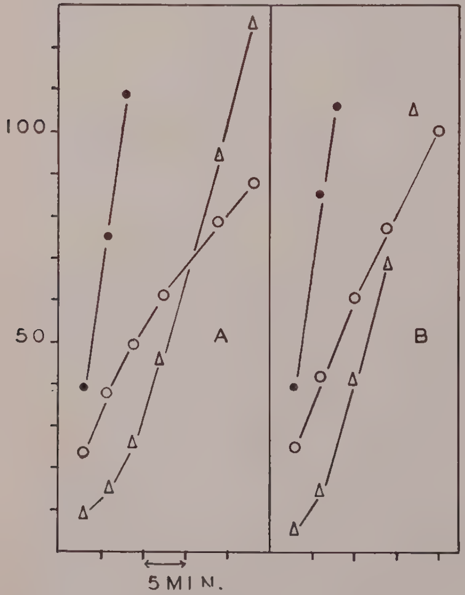

FIG. 6.

Shows effect of different amounts of gelatin on aerosol OT activated enzyme. Otherwise same as Fig. 1. A = enzyme + gelatin + substrate in proportion 1:1:2. B = enzyme + gelatin + substrate in proportion 1:3 1/3:2.

ture of Nelson and Dawson[2] would, therefore, possess both cresolase and catecholase activity. Variations in the degree to which these substrates are oxidized are evident in the different samples of protyrosinase even when activated by the same reagent (Fig. 1 to 6). The general reactions shown will be discussed under the results for the various activators employed.

Sodium dioctyl sulfosuccinate (aerosol OT), the anionic detergent most extensively employed in work on the activation of protyrosinase, produces a tyrosinase strikingly active for tyramine-HCl and about equally active toward p-cresol and catechol (Fig. 1). Experiments carried out using the relative proportions of reagents as suggested by Adams and Nelson[9] show little difference in results from those produced by the author's methods (Fig. 1). Addition of gelatin to the reaction mixture has little effect upon the enzyme's activity (Fig. 1, 6). For aerosol OT activated tyrosinase one might reasonably conclude that no marked differences in its activity for monohydric and o-dihydric phenols exist.

Sodium dodecyl sulfate activation produces a tyrosinase with properties closely resembling those for aerosol OT (Fig. 2). All 3 substrates are oxidized but with no marked or significant differences in favor of any of the three. Results are practically identical regardless of the method used in carrying out the experiments (Fig. 2).

$HgCl_2$ activated enzyme shows a marked monohydric phenol activity and a correspondingly low value for a o-dihydric substrate. This perhaps might be termed an enzyme high in cresolase activity and low in catecholase activity. Results obtained by the Adam and Nelson[9] method give similar values (Fig. 3).

Urea activated tyrosinase closely resembles that produced by $HgCl_2$ in showing low o-dihydric activity (Fig. 4). Recently, marked similarities in the resistance of the tyrosinase produced by $HgCl_2$ and urea to temperature and $HgCl_2$ have also been pointed out.[5, 10]

Activation of protyrosinase by chemical means is more or less open to the objection that the chemical reagents themselves produce, in addition to activation, other secondary chemical changes which in turn lead to erroneous conclusions.

Activation by heat (70°C-10′) affords a rather unique method for comparing the effects of chemical and physical reagents upon protyrosinase. Fig. 5 shows graphically the action of heat activated tyrosinase on the three substrates. A rather wide range in rates of reaction occurs. No marked differences in behavior of heat activated samples of tyrosinase over those produced by purely chemical means seem evident.

Adams and Nelson[9] observed rather marked effects of inert proteins like gelatin upon the inactivation of their enzyme. When added to tyrosinase from the grasshopper egg no significant results were noted (Fig. 6).

Discussion. That tyrosinase from either plant or animal sources varies to a great degree in its activity towards monohydric and o-dihydric phenols seems well established (Nelson and Dawson[2]). Tyrosinase from different species of plants has been shown by the work of Nelson and his students[2] to be greatly modified in its final catalytic properties by the methods employed in its isolation and purification. In the present experiments, tyrosinase from the one species of grasshopper, when produced through activation by different reagents, also shows marked variations in its catalytic responses to different substrates. Some evidence of these changes has recently been shown in results of experiments dealing with the effects of synthetic detergents on the protyrosinase of grasshopper eggs.[6] The degree to which differences in activity of plant tyrosinase are produced by chemical means appears greater than that found for the enzyme from the grasshopper egg.

Summary. 1. Protyrosinase from the egg of the grasshopper, *Melanoplus differentialis* was activated to form tyrosinase by the following reagents: sodium dioctyl sulfosuccinate (aerosol OT), sodium dodecyl sulfate, $HgCl_2$, heat (70°-10′), and urea. 2. Tyrosinase, produced by different activators, was allowed to act upon monohydric (tyramine-HCl, p-cresol) and o-dihydric (catechol) phenols as substrates and relative rates of activity studied. 3. All enzymes tested showed activity for both monohydric and o-dihydric phenols. 4. Urea and $HgCl_2$ activated tyrosinase showed high monophenolase activity and low catecholase activity. All other enzyme samples exhibited both monophenolase and catecholase activity in varying degrees. 5. Animal and plant tyrosinases seem similar in that both possess enzymatic activities more or less conditioned by chemical treatments.

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[10] Bodine, J. H., and Tahmisian, T. N., *Arch. Biochem.*, 1943, **2**, 403.

14899 P

Prolongation of Hypnosis by Epinephrine and Insulin.

JOHN F. REINHARD. (Introduced by Erwin E. Nelson.)

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There is evidence that analgesic agents prolong sleep induced by hypnotic drugs.[1,2] That epinephrine or insulin might also potentiate hypnosis was suggested by reports which indicated that brain metabolism was retarded by a number of amine derivatives[3] and by insulin-induced hypoglycemia.[4] The purpose of this preliminary paper is to report that highly significant increases in sleeping times have been obtained in white mice pretreated either with epinephrine or insulin.

Method. Pretreatment consisted of injecting mice (20-30 g males) either with epinephrine (0.04 mg/kg, ip and im) or with insulin (0.05 U ip and im). Twenty minutes after pretreatment Evipal Soluble (100 mg/kg ip) was administered to the pretreated animals and to others which had received no pretreatment (controls). The duration of hypnosis was taken as the interval in minutes between the loss and the return of the righting reflex.

Results. The data have been summarized in Table I.

It will be noted that mice treated with epinephrine and Evipal slept approximately 1.5 times as long as those receiving Evipal alone (controls). The significance of the increases was tested by calculating the statistic *t*.[5] The *t* values given in Table I exceed by a wide margin those listed in Fisher's Tables[6] for $P = 0.001$; therefore, the observed increases in sleeping times are to be regarded as highly significant since the probability of their occurrence by chance is less than 1 in 1000. Although the dosage (0.04 mg/kg) was larger than that customarily used in human beings, it was less than 1% of the LD_{50} for the mouse as determined in these laboratories. Especially noteworthy is the fact that such potentiation occurred even though the hypnotic drug was not administered until 20 minutes after the injection of epinephrine. At this time the customary signs of epinephrine action were missing except for pilomotor activity which was definite.

That epinephrine prolonged the action of

TABLE I.
Potentiation of Evipal Hypnosis in Mice.*

			No. of animals		Avg. sleeping times (Min.)			
Pretreatment Drug	Dosage	Route of administration	Pretreated	Controls	Pretreated	Controls	Pretreated % of controls	*t* Values
Epinephrine	0.04 mg/k	Im.‡	10	20	59.2	31.4	188.5	15.418†
		"	29	29	56.0	33.6	166.7	13.835†
		Ip.	10	20	51.5	32.5	158.5	6.307†
		"	10	20	46.1	31.4	146.8	7.622†
Insulin	0.05 Units/ mouse	Im.	20	20	58.7	33.2	176.8	12.977†
		Ip.	20	20	47.0	32.5	144.6	6.631†
		"	16	20	61.7	34.7	177.8	12.242†

* Evipal Soluble, 100 mg per kilo ip 20 min after pretreatment.
† Highly significant.
‡ Injection made into the lateral muscles of the thigh.

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[1] Barlow, O. W., Climenko, D. R., and Homburger, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 11.

[2] Loewe, S., *J. Am. Pharm. Assoc.*, 1940, **29**, 162.

[3] Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, 1933, **27**, 1609.

[4] Himwich, H. E., and Fazekas, J. F., *Am. J. Physiol.*, 1937, **119**, 335.

[5] Fisher, R. A., *Statistical Methods for Research Workers*, London, 7th edition, 1938.

[6] Fisher, R. A., and Yates, F., *Statistical Tables for Biological, Agricultural and Medical Research*, London, 1938.

Evipal by locally retarding its absorption is untenable in view of the similarity of the results (Table I.) obtained for epinephrine by intraperitoneal and intramuscular routes of administration. The insulin-pretreated mice slept slightly longer than those treated with epinephrine. In the dosage employed (0.05 U) there were no convulsions on awakening or other untoward signs. These findings are of interest since the two agents are chemically unrelated and are both normally produced in the body. An extension of the present study is being planned to supply information regarding mechanisms of action. In this connection it is of interest to note that epinephrine has been reported to have an analgesic action in man and in animals.[7]

Summary. White mice pretreated either with epinephrine or insulin and subsequently given Evipal slept considerably longer than the control animals receiving only Evipal. The results are highly significant as determined by statistical analysis.

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[7] Ivy, A. C., Goetzl, F. R., Harris, S. C., and Burrill, D. Y., *Quarterly Bull. Northwestern University Medical School*, 1944, **18**, 298.

14900 P

Penicillin as a Prophylactic in Abdominal Surgery.*

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This investigation was undertaken to study the prophylactic value of penicillin in abdominal surgery by depositing penicillin† in the peritoneal cavity and determining the absorption rate of penicillin from the peritoneum into the blood stream.

Material and Method. Seven unselected cases were chosen for this experiment.

Case 1. Clean peritoneum, multiple fibroids. Supracervical hysterectomy and bilateral salpingo-oophorectomy.

Case. 2. Many pelvic adhesions, multiple fibroids. Supracervical hysterectomy and bilateral salpingo-oophorectomy.

Case 3. Ascites, tuberculous. Biopsy.

Case 4. Fibroid, enlarged uterus, 4 months gestation. Myomectomy and hysterectomy.

Case 5. Marked adhesions, old tubal infection, multiple fibroids. Supracervical hysterectomy and left salpingo-oophorectomy.

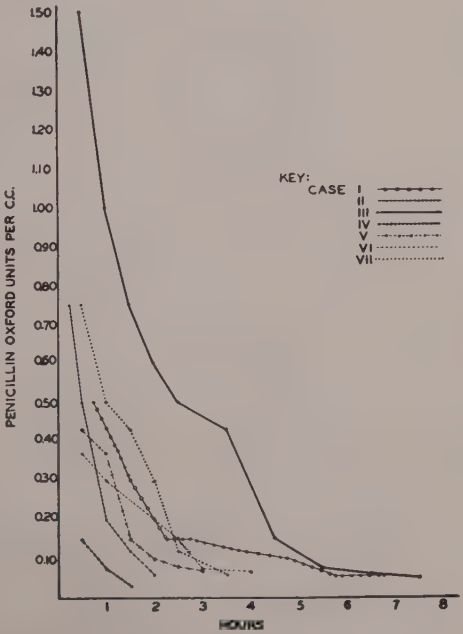

Fig. 1.

Blood level determinations after instillation of 100,000 Oxford units of penicillin into the peritoneal cavity.

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* Aided by grants from Friends of the Hospital and the Dazian Foundation for Medical Research.

† We are indebted to Mr. John L. Smith of the Charles Pfizer Company, Brooklyn, N. Y., who supplied the penicillin used in this study.

Case 6. Twisted pedunculated fibroid. Myomectomy.

Case 7. Ovarian cyst, clean peritoneum. Left cystectomy.

All these patients received at the end of the operation one hundred thousand Oxford units of penicillin in 20 ml normal saline solution instilled into the peritoneum. Blood was drawn half hourly and hourly up to 8 hours for serum penicillin assays in order to estimate its rate of absorption. The blood was allowed to clot and stored in the refrigerator over night. The penicillin levels were determined by the method of Rosenblatt, Alture-Werber *et al.*[1]

Results. It can be seen from Fig. 1 that the absorption of the instilled penicillin from the peritoneum into the blood stream takes place over a longer period and is more sustained than the intramuscular or the fractional intravenous routes. Peak levels are reached 30 minutes after instillation. Amounts of penicillin sufficient to combat penicillin sensitive organisms are found 3 (Cases 5, 6, 7) to 7 (Cases 1, 3) hours after administration. Case 3 with ascites and a tuberculous process attained the highest blood levels (1.5 Oxford units per ml of serum) and 0.05 Oxford units per ml were present even 7½ hours after the peritoneal deposition.

Summary. Penicillin given by the intraperitoneal route in a single dose of one hundred thousand Oxford units is detectable in the blood stream 3 to 7 hours in 5 cases, and one to 2 hours in 2 cases in sufficient amounts to neutralize penicillin sensitive invaders in abdominal surgery.

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[1] Rosenblatt, Philip, Alture-Werber, Erna, Kashdan, Florence, and Loewe, Leo, *J. Bact.*, 1944, **48**, 599.

14901

Chronic Oral Toxicity of Choline Chloride in Rats.*

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The demonstration of the ability of the rat to metabolize relatively large single doses of choline chloride[1] led to an attempt to discover what limits of tolerance exist for chronic administration. Two series of experiments were therefore carried out, (a) in which choline chloride at various levels was incorporated in the solid diet and (b) in which solutions of choline chloride were substituted for the drinking water.

Choline Chloride in Food. Choline chloride at levels of 0.01, 1, 2, 7, 5, and 10% of the diet was fed for periods of 3 to 4 months to groups of 5 rats each. The average body weight curves (Fig. 1) demonstrated that choline at 0.01 and 1% of the diet produced no effect on growth as compared to litter mate controls. 2.7% of choline reduced growth by about 20%, 5% of choline by about 45%, and 10% of choline in the diet permitted no growth.

Food intakes were measured daily for each group over an 8-week period (Table I., A). The lower growth rates in the groups on higher choline intakes were not a result of any specific toxic effect but were evidence of a refusal of food.

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* This work was supported in part by grants from the Carnegie Corporation of New York, from the Nutrition Foundation, and by grant No. 477 from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association.

This paper was presented in part at the meeting of the American Chemical Society, Pittsburgh, September, 1943.

[1] Hodge, H. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 26.

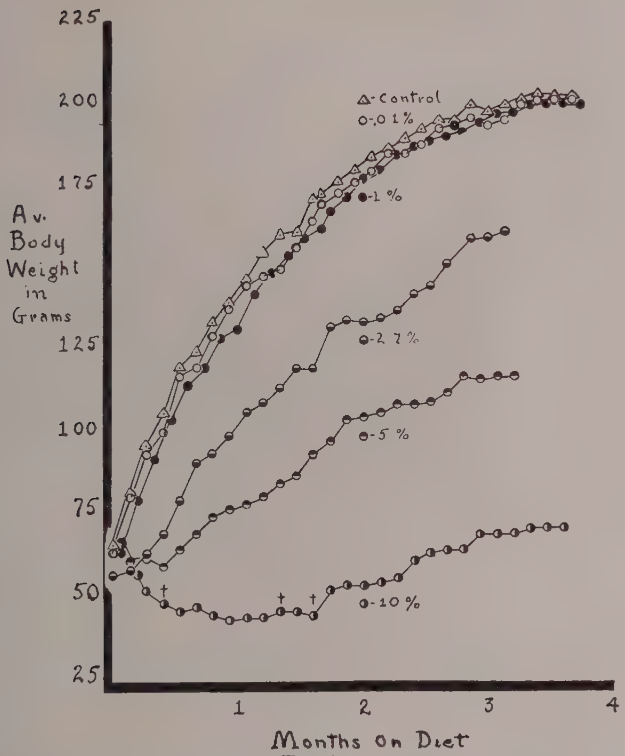

FIG. 1.

Growth curves of groups of rats on various dietary levels of choline chloride. Little effect on growth was observed in diets of 0.01 and 1.0%; progressively greater effects were obtained by including 2.7, 5, and 10% of the drug in the diets. Each + sign marks the death of one rat.

At the end of the experimental period the rats were autopsied. The organs from each rat were weighed and samples of a number of tissues were taken for histological examination. Although the smaller rats (those on the higher choline levels) had smaller organs, the average organ weights per gram rat were comparable for all groups except for the rats whose diet contained 10% choline. In these rats on the highest dosage, the organs (except the spleen) were all much *larger* per gram rat than is normal. In the rats on high choline intake almost no fat was seen either in the subcutaneous tissues, the mesentery, or the perirenal sites. The absence of fat was probably a result of the low food intake; however, the lipotropic action of choline[2] may also have been partly responsible.

The results of the histological examination conducted by Drs. J. R. Carter and H. Kattus of the Department of Pathology were largely negative. In addition to sections of the brain, stomach, kidney, heart, lung, spleen and liver, sections were also prepared of small intestine, large intestine, and ovary or testis. In the group receiving 0.01% of choline, the tissues were essentially normal. In the group receiving 1% of choline, there was a tendency toward an increase in the spleen of hemosiderin-containing phagocytes; this pigment was separately identified by special iron stains. In this group, one rat kidney showed prominent areas of lymphoid infiltration. In the liver of one rat of the group receiving 2.7% of choline, was found an area of focal degeneration. In the other groups including the con-

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[2] Best, C. H., and Huntsman, M. E., *J. Physiol.*, 1932, **75**, 405.

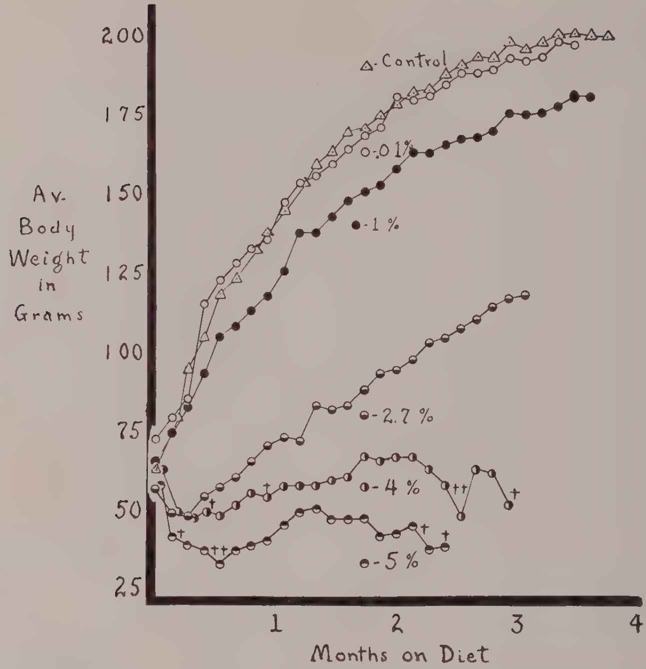

FIG. 2.

Growth curves of groups of rats drinking various solutions of choline chloride. A concentration of 0.01% produced little effect; however, increasing decrements in growth followed drinking 1.0 and 2.7% solutions. Rats given a 5% solution did not regain their original body weight during a period of over 2 months.

trol group, all sections were negative, although (particularly from rats on the higher choline intakes) the sections of many organs appeared smaller than the control tissues. In general, no consistent histopathological changes could be attributed to the inclusion of choline chloride in the food.

Choline Chloride in the Drinking Water. Choline chloride solutions containing 0.01, 1.0, 2.7, 4 and 5% by weight were substituted for the drinking water for the groups of 5 rats each whose growth curves are shown in Fig. 2. The lowest level as was found with the previous dietary experiment produced no diminution of the growth rate. However, drinking 1% choline solution produced a decrease in growth; this was in distinction to the normal growth rate of rats ingesting a 1% choline diet. A 40% reduction in growth was obtained when the rats drank a 2.7% choline solution. No growth occurred when they drank a 4% solution, and on a 5% solution, the survivors after 2 months weighed only 55 and 59%, respectively, of their weight at the start of the experiment. Other groups of rats were given solutions of 0.3, 3.0, 6.7 and 10% choline, respectively. These rats responded as would be expected from the effects seen in the groups described above. No rats drinking 6.7 or 10% choline solutions survived more than about 8 weeks and 7 of 10 rats on these levels died within 10 days.

The water consumption for each group was estimated daily during the first 8 weeks of the experiment (Table I., B). The growth curves reflected the intakes.

At the end of the experimental period the rats were autopsied and the organs treated (Table II., B) as before. The most noticeable consistent change in organ weights occurred in the livers; increasing percentages of choline gave smaller livers. However, when expressed on the basis of percentage of body weight, the average organ weights from the

TABLE I.
Food and Water Consumption by Rats Receiving Various Levels of Choline Chloride.

Choline Content %	A. Choline in diet Avg daily intake Initially g	A. Choline in diet Avg daily intake After 8 weeks g	B. Choline in water Avg daily intake Initially ml	B. Choline in water Avg daily intake After 8 weeks ml
0.01	8.7	12.6	21	33
0.1			14	21
2.7	4.5	12.3	7	12
5.0	3.3	7.3	2	4.5
10	2.1	2.5		

various groups were fairly comparable, except for the group receiving the 3% choline solution. Here the tendency was for the organ weights to constitute larger fractions of the body weight.

The histopathological examinations were carried out as previously described. In general the findings were negative. In the group receiving 0.01% choline, the spleens seemed to show an increase in the hemosiderin-containing phagocytes. In this group, 3 of 5 kidney sections showed in the cortex and pyramids a few focal collections of lymphocytes, most of which appeared to have a peri-arterial distribution. With these few exceptions, for all the other groups including the control rats the tissues were essentially normal. Again it may be said that no consistent pathological findings were observed which might reasonably be attributed to the effect of choline chloride.

Summary. Chronic feeding of a diet containing up to 1% of choline chloride produced no evidence of toxicity. Except for growth rate depression, no toxic effects of higher dietary levels have been observed. Rats which drank water containing 1% of choline chloride showed perceptible diminution in growth rate. Greater concentrations than 3% choline in the drinking water were poorly tolerated. No histopathological effects could be directly attributed to the ingestion of these amounts of choline.

TABLE II.
A. Average Weights of Various Organs of Rats Receiving Diets Containing Various Levels of Choline Chloride. (5 Rats in Each Group)

Choline HCl in diet %	Avg body wt at sacrifice	Average organ weights in grams Brain	Stomach	Kidney	Heart	Lung	Spleen	Liver
0.01	202	1.82	0.98	1.55	0.79	1.35	0.89	6.73
1.0	200	1.64	1.03	1.58	0.70	1.03	0.51	6.58
2.7	162	1.58	1.08	1.18	0.50	0.93	0.64	5.51
5.0	118	1.59	0.90	0.99	0.46	0.63	0.44	3.86
10.0	72	1.38	0.77	0.96	0.43	0.53	0.28	3.12
Control	202	1.59	1.11	1.68	0.62	1.11	0.59	6.54

B. Average Weights of Various Organs of Rats Receiving Various Concentrations of Choline Chloride in the Drinking Water. (5 Rats in Each Group)

Choline HCl in diet %	Avg body wt at sacrifice	Average organ weights in grams Brain	Stomach	Kidney	Heart	Lung	Spleen	Liver
0.01	199	1.65	1.05	1.60	0.67	2.02	0.64	6.27
0.3	165	1.51	0.96	1.55	0.63	0.98	0.50	5.72
1.0	183	1.70	0.90	0.90	0.70	1.10	0.70	5.10
2.7	121	1.50	0.80	1.30	0.50	0.70	0.40	4.10
3.0	76	1.46	0.70	0.98	0.36	0.67	0.25	3.46
Control	188	1.55	0.99	1.52	0.55	1.00	0.70	8.69

14902

Inhibition of Diabetogenic Action of Alloxan.*

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(Introduced by George R. Minot.)

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Administration of alloxan to animals results in marked changes in blood sugar[1-5] and in selective destruction of the islets of Langerhans.[3-5] The literature describing confirmation of these observations has been summarized by Joslin[4] and by Kennedy and Lukens.[5] Certain chemical and biochemical actions of alloxan can be inhibited by various substances. Bernheim[6] observed that alloxan accelerates the oxidation of ethyl alcohol by surviving liver tissue and that 3,4-diaminotoluene, phosphate buffer, pyrophosphate, bisulfite, and cyanide inhibit this accelerating effect. Alanine, methionine, cysteine, hemoglobin, and the chloride and sulfate ions did not affect this action of alloxan. Alloxan solutions are converted to alloxanic acid salts rapidly by the addition of bicarbonate, according to Labes and Freisburger.[7] Jacobs[8] noted inhibition of the "peroxidase-like effect" of an alloxan and aldehyde mixture by hydrazine, hydroxylamine, cyanide, bisulfite, and cysteine, whereas alcohols and amino acids other than cysteine did not inhibit this effect. His experiments indicated also that alloxan reacts with reducing agents like paraphenylenediamine, benzidine, pyrogallol, and iodides.

An investigation was made of the effect of these and similar substances in preventing alloxan from producing changes in the blood sugar and selective destruction of the islets of Langerhans.

Method. After having determined the amount of alloxan necessary to produce diabetes, rabbits and rats were injected with one of the supposedly inhibitory substances a few minutes before the injection of alloxan.

Rabbits, fasted for from 18 to 24 hours, and rats were used as experimental animals. For the rabbit experiments the alloxan was dissolved in physiological saline solution and was injected intravenously as a single dose; except where noted it was injected immediately after the injection of the supposedly inhibitory substance. The rabbits were then fasted for 48 hours, and blood sugar determinations were made at varying times during the first 12 hours, at 24 hours, and at 48 hours after the injection of alloxan. Blood sugar determinations were made by a colorimetric method combining zinc deproteinization and ferricyanide reduction.[9] Rats were injected subcutaneously with single 300 mg/kg doses of alloxan,[10] and the supposedly inhibitory substances were injected intraperitoneally. Macroscopic examinations were made of the organs of all animals, and microscopic examinations were made of pancreas tissue in representative rabbits and in all rats. In many animals microscopic exminations were made also of the liver, kidneys, adrenals, gonads, thyroid, parathyroids, heart and lungs.

The *in vitro* "peroxidase-like effect" of a mixture of alloxan and aldehyde was deter-

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* Aided in part by a grant from The Diabetic Fund, Boston Safe Deposit and Trust Company, Trustees.

† Died Jan. 3, 1945.

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[2] Ecker, E. E., Kalina, R., and Pillemer, L., *Enzymologia*, 1939, **7**, 307.

[3] Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet*, 1943, **244**, 484.

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[5] Kennedy, W. B., and Lukens, F. D. W., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 143.

[6] Bernheim, F., *J. Biol. Chem.*, 1938, **123**, 741.

[7] Labes, R., and Freisburger, H., *Arch. exp. Path. u. Pharmakol.*, 1930, **156**, 226.

[8] Jacobs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 131.

[9] Weinglass, A. R., and Taylor, F. H. L., in preparation.

[10] Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **245**, 384.

mined according to the method described by Jacobs.[8] A few drops of fresh solutions of alloxan and formaldehyde were mixed with small amounts of various substances, and the mixture diluted to 5 cc with distilled water. Then a few drops of hydrogen peroxide and of an acetic acid solution of benzidine were added, and the solution was observed for the development of blue color.

Results. Single intravenous injections of alloxan in doses of 150 mg/kg caused the usual blood sugar changes and islet damage[4] in 48 of 50 control rabbits; doses of 175 mg/kg were effective in 15 of 16 rabbits. When the alloxan was injected immediately following administration of 3.4-diaminotoluene, orthophenylenediamine or sodium bisulfite the usual hypoglycemic and diabetogenic effects, as shown by blood sugar determinations and histological examinations of the process, did not occur (Table I.) The substances that did not inhibit noticeably these alloxan effects even when they were used in doses approaching lethal quantities were: sodium bicarbonate, sodium phosphates (pH approximately 7.0), sodium pyrophosphate, sodium cyanide, phenylhydrazine, hydroxylamine hydrochloride, cysteine hydrochloride, paraphenylenediamine, sodium pyrogallol, and lysine hydrochloride. The maximum dosages, in mg/kg, of the non-inhibiting substances given intravenously to rabbits were: sodium bicarbonate 500, sodium phosphate 500, sodium pyrophosphate 40, sodium cyanide 10, paraphenylenediamine 108, and lysine hydrochloride 180. The maximum dosages of substances given intraperitoneally were: phenylhydrazine hydrochloride 20, and hydroxylamine hydrochloride 10. The maximum dosage of cysteine hydrochloride was 500 mg/kg given subcutaneously.

When 120 mg/kg or more of diaminotoluene were injected within a period of 5 minutes before or after the injection of 175 mg/kg doses of alloxan, 19 rabbits did not show the usual blood sugar and islet changes following from the administration of alloxan alone. Administration of less than 100 mg/kg doses of diaminotoluene did not change appreciably the usual blood sugar and islet disturbances in 7 rabbits following simultaneous injection of alloxan. When the interval between diaminotoluene and alloxan injections was 15 min or longer, the usual blood sugar and islet cell changes resulting from alloxan administration occurred. In no animal were macroscopic or microscopic lesions of liver, heart, thyroid, parathyroids, adrenals, gonads, kidneys and lungs observed that could be attributed to the effects of diaminotoluene.

After injection of orthophenylenediamine in doses of 108 mg/kg or more, 9 rabbits that received 150 mg/kg doses of alloxan did not show the usual hypoglycemia, hyperglycemia or marked islet cell changes. One rabbit injected with a 54 mg/kg dose of orthophenylenediamine immediately before the 150 mg/kg dose of alloxan died with hypoglycemic convulsions and showed marked necrosis in all the islets. In none of these rabbits were lesions observed that could be attributed to the effects of the orthophenylenediamine on macroscopic and microscopic examinations of the liver, kidneys, heart, and adrenals.

After injection of sodium bisulfite in doses of 80 to 130 mg/kg 8 of 13 rabbits did not develop the usual fatal hypoglycemia and marked islet necrosis usually resulting from the 150 mg/kg dose of alloxan. No histologic changes were observed in liver, kidneys, heart, or adrenals that could be attributed to the effects of sodium bisulfite administration. None of the rabbits that survived the first 48 hours after injection of alloxan and diaminotoluene, orthophenylenediamine, or sodium bisulfite developed hyperglycemia afterwards.

On microscopic examination the sections of pancreas from rabbits receiving adequate doses of 3,4-diaminotoluene, orthophenylenediamine, or sodium bisulfite within 5 min of the alloxan administration showed no lesions (Table I.). In the remainder of the series the pancreas showed either necrosis of all islets, necrosis of most cells in scattered islets with other islets that looked normal, or necrosis of scattered cells in some islets in which many other cells looked normal.

The 300 mg/kg dose of alloxan caused marked islet cell necrosis in all of the control rats and in the ones treated with paraphenylenediamine, sodium pyrogallol, potassium iodide, sodium sulfite and sodium metabi-

TABLE I.
Effects of Inhibiting Agents on Diabetogenic Action of Alloxan.

No. of Rabbits	Dose of alloxan mg/kg	Inhibiting substances*	Dose mg/kg	Animals with changes in blood sugar: No. of rabbits	Hypoglycemia: Fatal	Hypoglycemia: with recovery	No change	Animals with necrosis in islets: All islets	Scattered islets	Scattered cells	No lesions	Not examined
1	250	3,4-diaminotoluene	200	1	0	0	1	0	0	0	1	0
1	200	"	250	1	0	0	1	0	0	0	1	0
2	195	"	250	1	0	0	1	0	0	0	1	0
		"	230	1	0	0	1	0	0	0	1	0
19	175	"	240	1	0	0	1	0	0	0	1	0
		"	230	1	0	0	1	0	0	0	1	0
		"	210	1	0	0	1	0	0	0	1	0
		"	200	7	0	0	1	0	0	5	0	2
		"	200†	1	0	0	1	0	0	1	0	0
		"	120	4	0	0	4	0	0	2	0	2
		"	240‡	1	1	0	0	1	0	0	0	0
		"	210§	1	1	0	0	1	0	0	0	0
		"	240‖	2	2	0	0	2	0	0	0	0
8	150	"	30	2	2	0	0	1	0	0	0	1
		"	61	2	2	0	0	0	0	0	0	2
		"	73	1	1	0	0	1	0	0	0	0
		"	98	2	2	0	0	2	0	0	0	0
		"	244	1	0	0	1	0	0	0	0	1
9	150	Orthophenylenediamine	108	6	0	0	6	0	0	3	0	3
		"	130	2	0	0	2	0	0	0	1	1
		"	54	1	1	0	0	1	0	0	0	0
13	150	sodium bisulfite	80	2	1	0	1	0	0	0	1	1
		" "	90	1	0	0	1	0	0	0	1	0
		" "	104	3	0	1	2	0	1	0	0	2
		" "	115	3	2	0	1	0	0	0	1	2
		" "	125	3	0	0	3	0	0	0	1	2
		" "	130	1	0	0	1	0	0	0	1	0

* injected intravenously
† " 5 min. before alloxan
‡ " 15 " after "
§ " 30 " " "
‖ " 15 " before "

sulfite.

The *in vitro* "peroxidase-like effect" of alloxan and aldehyde[8] was inhibited by thiourea, sodium thiosulfate, and sodium sulfite, but large doses of these substances failed to inhibit the diabetogenic action of alloxan injected simultaneously or immediately afterwards; sodium metabisulfite administration also did not affect noticeably the usual alloxan effects. The maximum dosages, in mg/kg, given intravenously to rabbits were: thiourea 300, sodium thiosulfate 680, sodium sulfite 120, and sodium metabisulfite 100.

Discussion. In flavin synthesis alloxan unites with orthodiaminodimethylbenzene, and it is thought that 3,4-diaminotoluene reacts similarly with alloxan. A yellowish coloration observed along the ear veins of the rabbits after injection of the diaminotoluene and alloxan indicates that this reaction may occur in the blood of these animals. A similar reaction may explain the protective effect of orthophenylenediamine. Apparently most of the alloxan is removed by such reactions before the alloxan can damage the islet cells irreversibly. The work of other investigators[11, 12] suggests that this damage occurs within the first 5 minutes after intravenous injection of alloxan. Failure of the diaminotoluene to protect against the alloxan when the interval between injections of the two substances was greater than 5 min is confirmatory evidence for this suggestion.

Bisulfite also inhibits the diabetogenic action of alloxan, apparently by combining with it.[6] This protective action was not demonstrable consistently unless almost lethal doses of 125 mg/kg or more of sodium bisulfite were injected. An explanation of the failure of other compounds that combine chemically with alloxan to inhibit its diabetogenic action is not available from these experiments. Lack of proper concentrations or pH conditions in the blood stream for some of these reactions to occur may be responsible in some cases.

Summary. It was attempted to inhibit the selective destructive effect of alloxan on islet cells and the resultant blood sugar changes by administration of substances that combine chemically with alloxan or that inhibit some of its chemical actions. Of these substances 3,4-diaminotoluene, orthophenylenediamine, and sodium bisulfite exhibited definite inhibitory effects.

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14903

Serological Reactions of Levans Synthesized from Sucrose and Raffinose by Bacterial Enzymes.*

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Two classes of polysaccharides, dextrans[1–5] and levans,[6–9] have been synthesized from sucrose through the action of cell-free enzymes of bacterial origin. In the case of the leuconostoc dextrans, the enzymatically synthesized polysaccharides were shown to have serological properties similar to those of the polysaccharides formed in living cultures of the bacteria from which the enzymes came. A demonstration of the same feature for the enzymatically synthesized levans at first appeared impracticable because there were no data in the literature to indicate any serological reactivity on the part of the "natural" levans

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* Aided by a grant from the Sugar Research Foundation.

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[2] Hehre, E. J., and Sugg, J. Y., *J. Exp. Med.*, 1942, **75**, 339.

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TABLE I.
Serological Reactions of Products Synthesized from Sucrose and Raffinose by the Cell-Free Enzymes.

		Highest dilution of enzyme-substrate mixture that gave precipitation in tests with different antiserums			
Source of enzyme	Substrate	*Streptococcus salivarius* antiserum	*Bacillus N9* antiserum	Type 2 pneumococcus antiserum	Control serums
Streptococcus salivarius	sucrose	1,000	1,000	5	0
	raffinose	400	400	0	0
	other sugars	10*	10*	0	0
	buffer solution	10*	10*	0	0
Bacillus N9	sucrose	1,000	1,000	0	0
	raffinose	1,000	1,000	0	0
	other sugars	5*	5*	0	0
	buffer solution	5*	5*	0	0
Leuconostoc mesenteroides	sucrose	0	0	10,000	0
	buffer solution	0	0	10*	0

0 = no precipitation with 1:5 dilution of the antigen.
* Represents reactive material contained in the original enzyme solution, which did not increase in amount during the incubation of the test mixtures.

formed in bacterial cultures. However, upon investigation we[10] found that the levans from sucrose cultures of *Streptococcus salivarius* and of a spore-forming bacillus gave precipitation and complement fixation when tested against antiserums produced by immunization with bacteria obtained from sucrose cultures of either species. Experiments were then made to determine if the products synthesized from sucrose or raffinose by cell-free enzymes would have serological properties similar to those possessed by the levans produced in the cultures.

Sterile cell-free enzyme solutions were prepared[2] from sucrose broth cultures of the strains of streptococcus and bacillus which had been utilized in the previous[10] study. Mixtures were made of enzyme plus sterile 0.2 molar solutions of the following sugars buffered with acetate at pH 5.8; sucrose, raffinose, glucose, fructose, glucose and fructose, maltose, lactose, trehalose, xylose, arabinose. In order to compare levan-formation with dextran-formation a mixture of sucrose plus leuconostoc enzyme was included. After incubation for 10 days at 23°C under aseptic conditions, all the mixtures were examined for opalescence, for material precipitable in 65% alcohol, for free reducing sugars, and for serological reactivity. Three sorts of serum were included: (1) antiserums of *Streptococcus salivarius* and of the bacillus, produced by immunization with sucrose-grown bacteria and known to be highly reactive with the levans from cultures of the streptococcus and the bacillus; (2) type 2 antipneumococcus serum known to be highly reactive with bacterial dextrans but entirely non-reactive with levans; (3) types 1 and 8 antipneumococcus serums known to be non-reactive with either dextrans or levans, which served as routine controls. The serums were diluted 1:12 and the antigen-serum mixtures were observed for precipitation after 1 hr at 37°C.

Results. In the mixtures containing sucrose and raffinose the enzymes of the streptococcus and bacillus brought about the development of opalescent material that was precipitable in 65% alcohol and also an accumulation of free reducing sugars; neither of these changes occurred in the mixtures of the enzymes with the other substrates. The physical and chemical

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[5] Hehre, E. J., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 240.

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[9] Hestrin, S., and Avineri-Shapiro, S., *Biochem. J.*, 1944, **38**, 2.

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changes in the sucrose and raffinose mixtures, and also the substrate specificity, are characteristic of levan-formation when it occurs in bacterial cultures, and are the same as those already reported[8] for the synthesis of levan by enzymes from species of *Aerobacter* and *Bacillus*.

The new fact (Table I.) is that the products formed from sucrose and raffinose by the streptococcus and bacillus enzymes had serological properties similar to those previously[10] found for the levans obtained from cultures of those bacteria. That is, the products of enzyme origin, like the levans of culture origin, had the capacity to cross-react with the antiserum of the other species as well as the capacity to react with the antiserum of its own species. This agreement in respect to the reactivities against the heterologous as well as the homologous antiserums, furnished better evidence of serological likeness between the products of enzyme and culture origins than would be furnished if the comparison were based only upon the reactivities against the antiserums of the homologous species.

The products synthesized by the streptococcus and bacillus enzymes were serologically different from those of the dextran products synthesized by the leuconostoc enzyme. The latter gave strong reactions with the type 2 pneumococcus but did not react with the two anti-levan serums, whereas, the opposite relationship was shown by the streptococcus and bacillus products. (The streptococcus enzyme did produce from sucrose a small amount of material reactive with the type 2 antiserum, but this minor exception was to be expected: a small but definite amount of dextran is formed in sucrose broth cultures of this as well as of some other strains of *S. salivarius*,[11] so that enzyme solutions prepared from them would likely contain some dextran-synthesizing enzyme along with a larger amount of levan-synthesizing enzyme.)

In addition to the tests made with the crude enzyme-substrate mixtures, experiments were made with material obtained by precipitation of a sucrose-streptococcus-enzyme mixture with 2.5 volumes of alcohol. The material was levo-rotatory and non-reducing but yielded abundant amounts of reducing sugars after mild acid hydrolysis (15 min at 60°C with 0.8N HCl); the value for fructose obtained in analyses by the Roe[12] procedure was 95% of that theoretically expected for levans. These data indicate that the enzyme derived material, although not pure, consisted chiefly of levan. When tested serologically, it gave precipitation in a dilution of 400,000 and complement fixation in a dilution of 1 million with the bacillus as well as the streptococcus antiserum, which represents essentially the same degree of serological reactivity as that shown by the purified levan of streptococcus culture origin.[10]

Summary. Cell-free, levan-synthesizing enzymes have been prepared from *Streptococcus salivarius* and from a spore-forming bacillus. The polysaccharide products formed by the action of these enzymes upon sucrose and raffinose possess serological properties similar to those recently described for the purified levans isolated from sucrose broth cultures of the living bacteria.

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14904

I. Stomal Ulcer Attending Complete Intragastric Regurgitation Influenced by Length of Afferent Duodenojejunal Loop.*

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Intragastric regurgitation of the contents of the duodenal loop has been looked upon as a desirable factor in gastric resection for ulcer. Schmilinsky[1] suggested placement of the afferent duodenojejunal loop in the Billroth II type of gastric resection back onto the stomach in such a manner that complete drainage of all the duodenal contents occurred back into the stomach. He termed this arrangement an "internal pharmacy" for neutralization of gastric acidity. McCann[2] reported that he had produced gastrojejunal ulcer in 80% of 26 dogs operated upon according to the Schmilinsky plan. A number of other investigators, Ivy and Fauley,[3] Weiss, Graves and Gurriaran,[4] Graves,[5] Maier and Grossman,[6] and Wangensteen and his associates,[7] repeated the McCann experiment with rather indifferent results. None of these investigators was able to confirm McCann's observations of a high incidence of gastrojejunal ulcer following complete drainage of the duodenal loop back into the stomach. Wangensteen and his associates indicated that disastrous results attended performance of the Schmilinsky operation on man and suggested that constant regurgitation of the duodenal loop content back into the stomach might stimulate the second or gastric phase of gastric secretion interminably. Kesavalu and Mann[8] have shown, in dogs with isolated gastric pouches, that the Schmilinsky procedure definitely enhances secretion from the pouch. This study was undertaken with the hope that some of the conflicts and confusions hedging about this problem could be resolved.

Methods of Study and Results. A total of 17 dogs was studied. The Schmilinsky-McCann operation of complete intragastric return of the entire content of the duodenal loop was performed in each animal. In the first series of 11 dogs the operation was accomplished in the following manner: These dogs were anesthetized and under aseptic conditions a laparotomy was performed. The pylorus of the stomach was excised. The duodenal end was then closed and inverted in the usual fashion by means of interrupted cotton sutures. At distances of 8 to 15 cm from the inverted duodenal stump, the intestine was transected. The proximal transected intestine was anastomosed onto the stomach. Thereby, complete intragastic regurgitation of the duodenal contents including bile and pancreatic juice was assured. The end of the distal loop of intestine was closed and inverted. A gastrojejunostomy, end-to-side was then performed between the end of the stomach and the side of the distal transected intestine.

Following operation convalescence was rapid. After a brief period of time, normal activity was assumed and appetite regained. At various intervals from 72 to 360 days these

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* The researches upon which this presentation is based were supported by the Augustus L. Searle Fund for Experimental Surgical Research, the Citizens' Aid Society and by grants of the Graduate School of the University of Minnesota.

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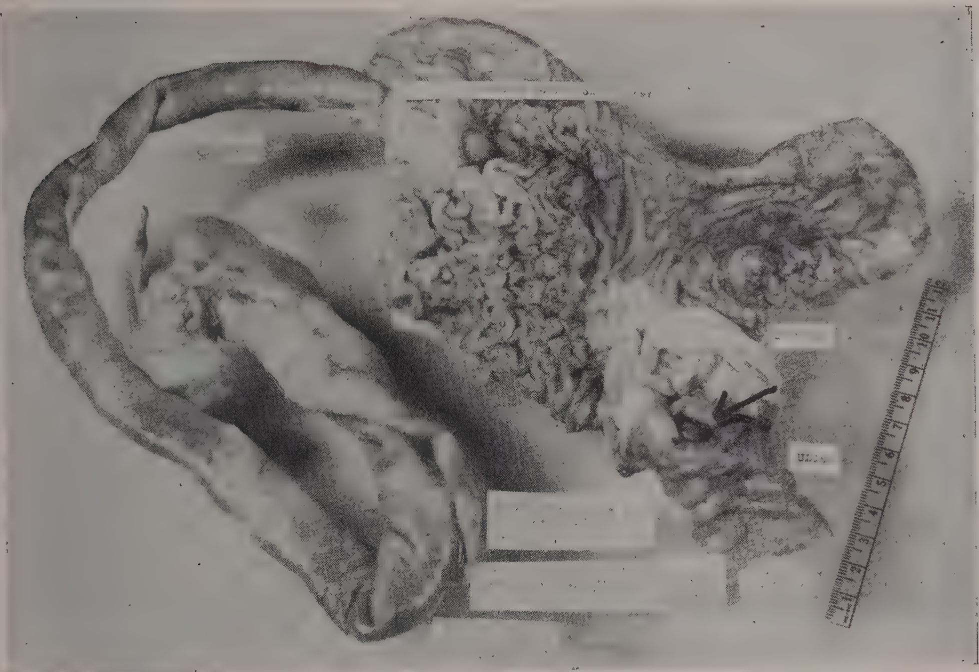

FIG. 1.
(Dog No. 132—Table II.) Photograph of long afferent duodenojejunal loop (76 cm), stomach and perforated stomal ulcer just beyond efferent outlet. (Ulcer at arrow).

animals were sacrificed. The results are recorded in Table I.

In the 11 dogs in which a short proximal duodenal loop was employed in the Schmilinsky-McCann procedure, gastrojejunal ulcer occurred only once (9.1%).

A second series of 6 dogs having the same operation subsequently was studied. The operation was identical in all details with that described in the first series of animals with one exception. In the first series the intestine was transected a short distance from the inverted duodenal stump. Thus, a short proximal loop was obtained. However, in this second series of dogs, the transection of the intestine was carried out at a lower level. The length of the proximal loop from the inverted or "blind" duodenal end varied from 76 to 90 cm. The transplantation of the proximal loops was high on the stomach in some instances, low in others.

In this second series, a short period of normal response was noted. As time progressed, however, the dogs became irritable, anorexic and languid; coma and death followed.

The results are tabulated in Table II.

TABLE I.
Occurrence of Gastrojejunal Ulcer in the Schmilinsky-McCann Procedure Employing a Short Afferent Duodenal Loop.

No. of dogs	Operation	Avg. length of proximal loop	Time of sacrifice of dog	Results
11	Excision of pylorus. Duodenal end closed. Intestine transected 8-15 cm from duodenal closure. Gastrojejunostomy made with distal intestine anastomosed onto stomach.	10 cm from the inverted duodenal stump.	72 to 360 days	One dog developed a gastrojejunal ulcer.

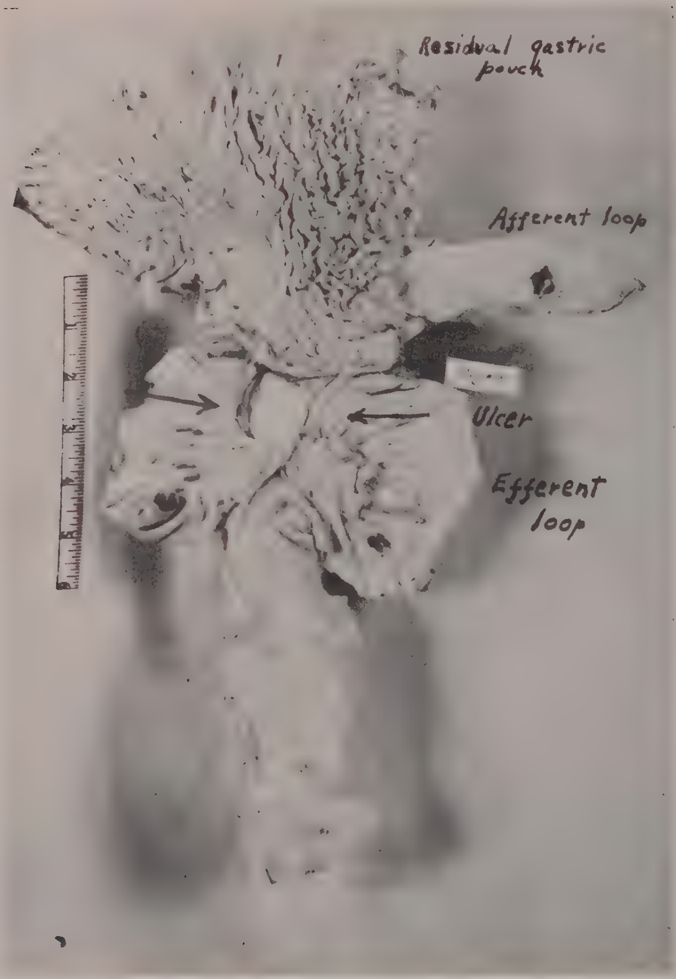

FIG. 2.
(Dog No. 84—Table II.) Photograph of large perforated stomal ulcer. Length of afferent duodenojejunal loop 76 cm.

The average survival period was 79.7 days. The area of the transplantation (high or low) of the proximal loop onto the stomach, did not appear to alter the end result. The incidence of gastrojejunal ulcer in this series was 83.3% (5 out of 6 dogs). Four of the 6 dogs exhibited perforated peptic ulcers. (Fig. 1.) No animal was sacrificed. Histamine was not administered to any of the dogs in this study.

Comment. These results clarify the confusion in the literature, concerning the results of complete intragastric drainage of the duodenal loop in dogs. The results of the experiments reported herein suggest that, the divergent results obtained by previous investigators are explicable on the basis of the length of the afferent loop employed. Our first experiments in this study were begun in 1939 and the problem has been under constant scrutiny in the laboratory in the intervening years.

The agency through which the length of the afferent loop in the Schmilinsky procedure influences so definitely the occurrence of stomal ulcer is not apparent. One thing is clear

TABLE II.
Occurrence of Gastrojejunal Ulcer in the Schmilinsky-McCann Procedure, Employing a Long Afferent Duodenojejunal Loop.

Dog No.	Wt. lb.	Operation	Length of afferent loop from inverted duodenal end cm	Survival days	Results
79	39	Excision of pylorus, duodenal end closed. Jejunum transected 66 cm from ligament of Treitz. Gastrojejunostomy made with distal jejunum and proximal jejunum anastomosed high on stomach.	76	102	Large ulcer in gastric fundic wall; greater curvature 6.6 x 5.5 cm.
81	36	"	76	50	Severe gastritis; many scattered pinpoint erosions.
83	40	" Placement of proximal loop low on stomach.	76	56	Large sealed perforated ulcer just distal to efferent gastrojejunostomy.
84	52	"	76	65	Generalized peritonitis; perforated ulcer 2.7 x 3.3 cm immediately distal to efferent gastrojejunostomy.
132	38	Site of placement of proximal loop on stomach not indicated in operative record.	76	145	Generalized peritonitis; perforated ulcer in efferent gastrojejunal loop.
202	42	"	90	55	Generalized peritonitis; perforated ulcer in efferent gastrojejunal loop.

* The average overall length of the intestine from the duodenum to the ileocecal valve was 292 cm.

however: Exclusion of hydrochloric acid, the best physiologic stimulus for the secretion of pancreatic juice with high buffer value, from contact with the duodenal mucosa the segment of mucosa richest in secretin, affords a plausible explanation for the greatly increased incidence of stomal ulcer in the experiments in which the long afferent loop was employed. Other likely causes would appear to be: The wide distance of spatial separation of alkaline and acid digestive juices by employment of the long efferent loop—in other words, a type of Mann-Williamson operation; and finally, the often suggested increased susceptibility to injury by acid of successively lower segments of intestinal mucosa.

Conclusions. 1. The incidence of gastrojejunal ulcer in dogs attending complete intragastric regurgitation of the duodenal loop contents is dependent upon the length of the afferent duodenojejunal loop. 2. In 11 dogs in which complete duodenal drainage by means of a short afferent loop (8 to 15 cm) was established, only one dog developed a gastrojejunal ulcer, an incidence of 9.1%. 3. Five out of 6 dogs undergoing the same operation in which the proximal jejunal loop was long (75 to 90 cm) developed stomal ulcer, an incidence of 83.3%. Four of these died of generalized peritonitis secondary to perforated ulcers.

14905

II. Length of Afferent Duodenojejunal Loop in Gastric Resection, a Factor in Stomal Ulcer.*

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A high incidence of gastrojejunal ulcer has been reported from some clinics even after extensive gastric resection. The experience of this clinic with gastric resection for ulcer would suggest that the so-called intractable or incurable ulcer is a myth and that, if the criteria of a suitable operation for ulcer are satisfactorily met, recurrent ulcer will not follow. In this study, it is proposed to evaluate the importance, if any, of the length of the afferent duodenojejunal loop in protecting against stomal ulcer following extensive gastric resection in dogs.

Methods and Results. This group of experiments was carried out on 22 dogs in 3 series. An extensive gastric resection, carried out on the Billroth II plan of operation, was performed in each animal in the 3 series of experiments. The length of the afferent duodenal loop was short in Series I. In Series II and III the afferent duodenojejunal loop was long. Histamine in beeswax was administered to the dogs in Series I and II.

The operative procedure was accomplished in the following manner. The dog was anesthetized by means of intra-tracheal ether or intravenous nembutal, 15 mg per pound. Under aseptic conditions a laparotomy was performed. The duodenum was transected immediately distal to the pyloric sphincter. The duodenal end then was inverted. An extensive resection of the stomach was carried out, approximately 75% of the distal portion of the stomach being excised. The Hofmeister procedure was performed on the lesser curvature side. Gastro-intestinal continuity was completed by means of an end-to-side gastrojejunostomy. The abdomen was closed and the animal removed to his cage. Interrupted cotton sutures were used throughout.

During the early convalescent period, water and food were withheld. Parenteral 10% glucose was administered daily in distilled water or saline solution as indicated. After 48 to 72 hr, water and food by mouth were allowed. By the 10th postoperative day, the regular daily diet consisting of dog kibble, horsemeat and table scraps had been resumed on regular feeding schedules.

Series I. Eleven dogs were used. These dogs were subjected to an extensive gastric resection (75%). The gastrojejunostomy was performed as close to the inverted duodenal end as was technically feasible, the distance from the blind duodenal end varying from 12 to 15 cm. After this operative procedure, 3 months were allowed to elapse. Then 30 mg histamine base in beeswax, prepared after the method of Code and Varco[1] was injected intramuscularly each day. The injection was made into the back muscles at multiple sites in order to allow uniform absorption. Injections were made at 5 P. M. daily, following which food pans were removed. Water was allowed throughout the night. Feeding was reinstituted early the following morning. A total of 40 to 45 injections was carried out on each animal. There were no deaths. The animals were sacrificed after the last injection. The results are tabulated in Table I.

In spite of severe histamine stimulation, not one gastrojejunal ulcer was encountered. This is significant. (Table I).

Series II. The identical operation described above was performed, (extensive gastric resec-

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* The researches upon which this presentation is based were supported by the Citizens' Aid Society, the Augustus L. Searle Fund for Experimental Surgical Research and by grants of the Graduate School of the University of Minnesota.

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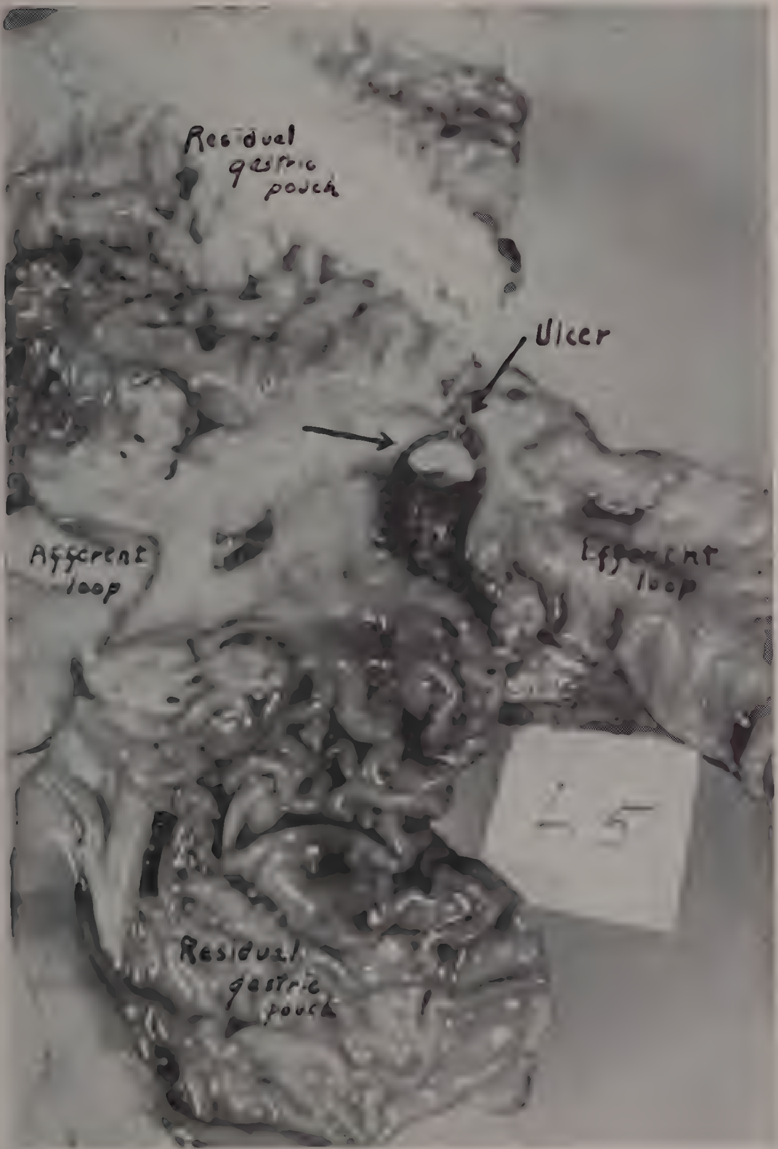

Fig. 1.
Photograph of perforated gastrojejunal ulcer occurring after extensive gastric resection (75%). The afferent duodenojejunal loop was 78 cm in length. Death occurred 12 days after the administration of histamine was commenced.

tion—75%) with one difference. In these animals, a longer afferent duodenojejunal loop was employed. The distance from the inverted duodenal end to the site of gastrojejunostomy varied from 27 to 78 cm. Similarly, a rest period of 3 months was allowed to intervene. Following this, 30 mg of histamine base in beeswax was injected intramuscularly daily. The results obtained are recorded in Table II.

In this series, gastrojejunal ulcer was ob-

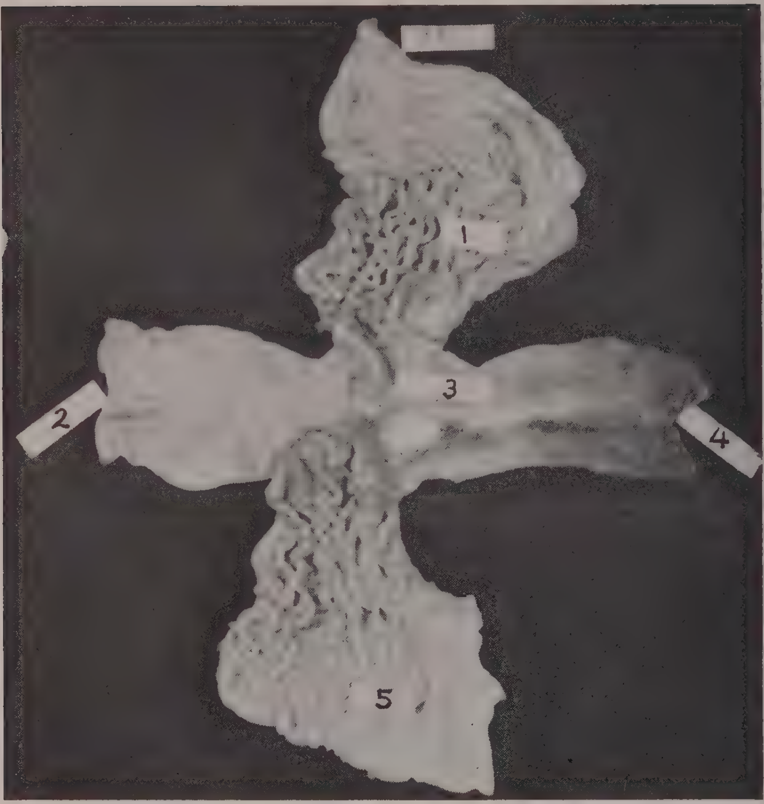

FIG. 2.
Spontaneous gastrojejunal ulcer occurring after a 50% gastric resection accompanied by a long afferent duodenojejunal loop. 1, stomach; 2, efferent loop; 3, jejunal ulcer; 4, afferent loop; 5, stomach.

served in each instance (100%). These results are in striking contrast to the results in Series I. Three of the 7 dogs in Series II died of generalized peritonitis attending perforation of a stomal ulcer. Dogs No. 4 and 5 had the longest afferent duodenojejunal loops. Both these dogs died of perforated gastrojejunal ulcer. Their survival periods were the shortest of the entire series. This occurrence suggests that a long afferent loop invites early formation of a gastrojejunal ulcer.

Series III. In a group of 4 dogs, gastric resection was done, varying in extent from 50 to 75%. The length of the afferent duodenojejunal loop in these 4 experiments varied between 78 and 144 cm. These dogs received no histamine. Two of the 4 dogs died of spontaneous perforation of a gastrojejunal ulcer located just beyond the efferent outlet. One dog, in which a 50% gastric excision had been done accompanied by an efferent duodenojejunal loop of 78 cm, was sacrificed 210 days after operation. There was no stomal ulcer. One other dog is still alive and apparently well more than 2 years after operation.

Summary. The results of these experiments are striking. In 11 dogs (Series I), with an extensive gastric resection (75%), in which the afferent duodenal loop was short (12 to 15 cm) stomal ulcer could not be provoked in a single instance by profound stimulation of gastric secretion with histamine in beeswax. In 3 of the 11 dogs, superficial gastric erosions were noted. In 7 dogs (Series II), in which

TABLE I.
Extensive Gastric Resection (75 per cent) with Short Afferent Loop.
Each dog was injected daily with 30 mg of histamine in beeswax.

Dog. No.	Wt. lb.	Operation	Avg. length of proximal duodenal loop	No. of injections	Survival days	Results
6	38	75% gastric resection with gastrojejunostomy	12 to 15 cm	40	40	No ulcer.
23	20	“		“	“	“ “
24	47	“		“	“	“ “ few small submucosal hemorrhages in gastric pouch.
28	27	“		“	“	No ulcer; small erosion adjacent to esophag.
46	26	“		“	“	No ulcer; few erosions in gastric pouch in juxtaposition to esophag.
48	35	“		“	“	No ulcer.
64	48	“		“	“	“ “
82	36	“		“	“	“ “
208	35	140 g of stomach resected.		45	45	“ “
242	22	130 g “ “ “		“	“	“ “
203	42	155 g “ “ “		“	“	“ “

the extent of the gastric resection was the same (75%), the only difference being that the afferent duodenojejunal loop was longer (27 to 78 cm), a gastrojejunal ulcer occurred following histamine stimulation in each instance. In a third series of 4 dogs with long afferent duodenojejunal loops which received no histamine after gastric resection varying in extent from 50 to 75%, 2 (50%) developed spontaneous perforated gastrojejunal ulcer.

Discussion. These results suggest definitely that the antecolic anastomosis with a long proximal duodenojejunal loop, even when accompanied by an extensive gastric resection,

TABLE II.
Extensive Gastric Resection with Long Afferent Duodenojejunal Loop.
Each dog was injected daily with 30 mg of histamine in beeswax.

Dog No.	Wt. lb.	Operation	*Length of proximal loop cm	No. of injections	Survival days	Results
244	25	75% gastric resection with gastrojejunostomy; 128 gm of stomach resected.	27	45	45	Large jejunal ulcer opposite the gastrojejunostomy.
246	35	“ ; 130 gm of stomach resected.	27	45	45	Large jejunal ulcer oppostie gastrojejunal stoma.
254	38	“ ; 160 gm of stomach resected.	42	22	22	Generalized peritonitis; perforated jejunal ulcer.
162	42	75% gastric resection; 170 gm of stomach resected.	50	48	48	Large irregular gastrojejunal ulcer 3 x 3.5 cm.
118	30	“ ; 128 gm resected.	54	48	48	Large excavated gastrojejunal ulcer 4 x 5.5 cm.
5	35	75% gastric resection with gastrojejunostomy.	78	12	12	Generalized peritonitis; opposite gastrojejunal stoma 4 x 3.5 cm.
4	34	“	78	3	3	Generalized peritonitis; perforated gastrojejunal ulcer in efferent loop.

* Average over all length of small bowel 310 cm.

TABLE III.
Extensive Gastric Resection (50 to 75 per cent). No Histamine Given.

Dog No.	Wt. lb.	Operation	Length of proximal loop cm	Survival days	Results
88	30	50% gastric resection with gastrojejunostomy.	78	*210	Sacrificed; no ulcer. (Over all length of small bowel 319 cm.)
98	25	“	78	420	Generalized peritonitis with perforated gastrojejunal ulcer. (Over all length of small bowel 323 cm.)
73	60	60% gastric resection.	144	338	Spontaneous perforation of a gastrojejunal ulcer with generalized peritonitis. The entire gastro-intestinal tract contained fresh and changed blood. (Over all length of small intestine 401 cm.)
74	48	75% gastric resection with gastrojejunostomy.	90	†	

* This dog was sacrificed. Neither an ulcer nor a fistula was found.
† Still living at 761 days, gastric resection done 12-16-42, negative exploration 1-30-45.

is not a satisfactory operation for ulcer in man. Man's small intestine is approximately twice the length of the small intestine in the dog. The length of the duodenum in man is stated by anatomists to vary between 25 and 30 cm. It has been common practice for some gastric surgeons to make the anastomosis 30 (Balfour)[2] or more (Lahey)[3] cm beyond the suspensory duodenojejunal ligament of Treitz. Kiefer[4] has reported a series of 173 extensive gastric resections for duodenal ulcer in which the incidence of gastrojejunal ulcer was 11.4%. In that series the antecolic long proximal duodenojejunal loop was employed in the anastomosis. In this clinic, a series comprising now more than 300 consecutive gastric resections, has been operated upon for ulcer, employing the criteria of a satisfactory operation for ulcer described elsewhere.[5–8] In this group only one stomal ulcer has developed. The 75% gastric resection, employing a short afferent duodenojejunal loop with a retrocolic anastomosis made at or just proximal to the suspensory duodenojejunal ligament of Treitz has been standard practice in operating upon patients for ulcer in this clinic for several years.

Conclusions. Extensive gastric resection (75%) in dogs when accompanied by a long afferent duodenojejunal loop is followed by a high incidence of spontaneous gastrojejunal ulcer. When histamine in beeswax is administered to such dogs, stomal ulcer occurs regularly. When gastro-intestinal continuity is reestablished by a short afferent duodenal loop in the Billroth II plan of operation, stomal ulcer after extensive gastric resection (75%) can not be provoked even when histamine in beeswax is administered.

It is obvious that a short afferent duodenal loop is an important component of a satisfactory operation when gastric resection on the Billroth II plan of operation is invoked in the surgical treatment of ulcer.

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[2] Eusterman, G. B., and Balfour, D. C., The Stomach and Duodenum. W. B. Saunders Co., Phila., 1935. Fig. 326.

[3] Lahey, F. H., and Marshall, S. F., *Surg. Gyn. and Obst.*, 1939, **69**, 498 (in legend of Fig. 14).

[4] Kiefer, E. D., *J.A.M.A.*, 1942, **120**, 814.

[5] Wangensteen, O. H., and Lannin, B. G., *Arch. Surg.*, 1942, **44**, 489.

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14906

On the Relative Toxicity of Nicotine and Nornicotine.

P. S. Larson, H. B. Haag and J. K. Finnegan.

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In recent years, Markwood[1] has called attention to the fact that certain strains of *Nicotiana tabacum* may contain relatively large amounts of nornicotine (a substance that differs chemically from nicotine only in that the nitrogen atom in the pyrrolidine ring is not methylated). While such "high nornicotine" strains probably constitute only a small minority of those generally used by man, we have recently shown[2] that on an average, tobacco of cigarette quality contains 15 to 20% as much nornicotine as it does nicotine. More complete knowledge concerning the toxicity of nornicotine therefore seems pertinent.

An earlier publication by us[3] included the following data pertaining to the toxicity of nicotine and nornicotine:

Nicotine: intraperitoneal LD 50 for mice = 10.3 mg per kg, for rabbits = 14 mg per kg; intravenous LD 50 for rabbits = 6.25 mg per kg.

Nornicotine: intraperitoneal LD 50 for mice = 21.7 mg per kg, for rabbits $>$13.7 mg* per kg; intravenous LD 50 for rabbits = 3.0 mg per kg.

From the above data it would appear that nornicotine is less toxic than nicotine by intraperitoneal administration and more toxic than nicotine by intravenous administration. This reversal in toxicity we ascribed to the difference in the dissociation constants of the two substances, a difference which would result in slower absorption of nornicotine as compared to nicotine following intraperitoneal administration. In other words, from the above data alone, it would seem that nornicotine is inherently more toxic than nicotine when factors of absorption are minimized. Recently we have obtained the following additional toxicologic data showing that as a generalization this is not valid:

Nicotine: intravenous LD 50 for mice = 0.55 mg per kg.

Nornicotine:† intravenous LD 50 for mice = 3.4 mg per kg.

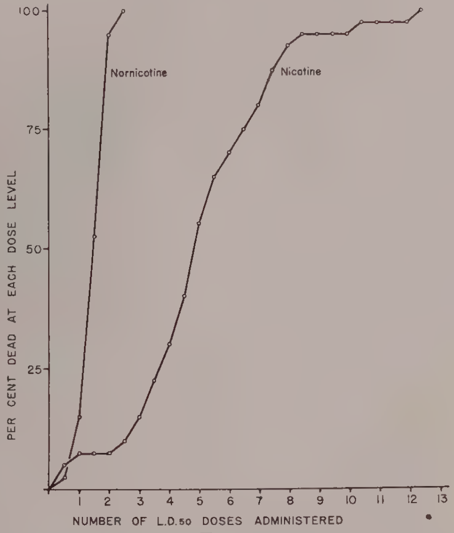

Fig. 1.

Toxicity curves for nicotine and nornicotine, administered intraperitoneally to mice in one-half LD$_{50}$ doses at 15-min intervals.

Thus, whereas the intravenous LD 50 for nornicotine is about equal for both species, the intravenous LD 50 values for nicotine show it to be definitely more toxic than nornicotine to the mouse and less toxic than nornicotine to the rabbit.

In view of this, it seemed of interest to compare the ability of the two species to detoxify nicotine and nornicotine. Following the technique employed by Weatherby[4] and administering intravenously one-sixth LD 50 doses at 2 min intervals to the point of death, we have previously shown[3] that the rabbit disposes of nornicotine at a rate comparable to that found for nicotine.[4] Since repeated intravenous injections into the mouse offer some difficulty, the comparison in this species was carried out as follows:

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[1] Markwood, L. D., *Science*, 1940, **92**, 204.

[2] Larson, P. S., and Haag, H. B., *Ind. and Eng. Chem. Analyt. Ed.*, 1944, **16**, 86.

[3] Larson, P. S., and Haag, H. B., *J. Pharm. and Exp. Therap.*, 1943, **77**, 343.

* The L.D. 50 is definitely greater than this value, but lack of sufficient nornicotine prevented a more exact estimation.

† Kindly furnished to us by Dr. R. C. Roark, United States Department of Agriculture, Bureau of Entomology and Plant Quarantine.

[4] Weatherby, J. H., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 593.

Employing 2 groups of 40 mice each, each mouse in one group was given ½ the LD 50 dose of nicotine intraperitoneally every 15 min until death, and each mouse in the second group was similarly treated with ½ LD 50 doses of nornicotine. The results are expressed graphically in Fig. 1. From this it is seen that the mouse detoxifies nicotine much more rapidly than it does nornicotine. In this respect then, the mouse again differs from the rabbit.

Summary. Whereas, the mouse and the rabbit as regards acute toxicity are about equally sensitive to nornicotine, they differ markedly in their sensitivity to nicotine as well as in their ability to detoxify the two substances. In view of such species variation it would seem impractical to evaluate the relative toxicity of nicotine and nornicotine for man on the basis of animal data. Since nicotine and nornicotine are quite closely related structurally, these results again emphasize the hazard of transferring to man toxicity data on related compounds obtained on one species of animal.

14907

Acute Nature of Alloxan Damage.*

GEORGE GOMORI AND MARTIN G. GOLDNER.
From the Department of Medicine, The University of Chicago.

Neutralization of an alloxan solution with NaOH completely abolishes its diabetogenic action.[1] Since alloxan, if injected intravenously, is bound to be neutralized by the buffers of the blood in a very short time, the conclusion that its toxic action must be limited to a few minutes seems to be logical. There are also indications that alloxan is not only neutralized but actually decomposed or disposed of in some way within a few minutes. Leech and Bailey[2] found that alloxan injected intravenously can be demonstrated in the blood for only 5 min after the injection.

That the toxic effect of alloxan takes place within a few minutes after injection could be proven by the following experiments:

Thirteen dogs were laparotomized under ether anesthesia, and that part of the pancreas which lies free in the duodenal mesentery was exposed, and its blood supply temporarily interrupted by placing two rubber-protected soft clamps along its both sides. Now a diabetogenic dose (60 to 100 mg/kg) of alloxan was injected rapidly into a leg vein. The clamps were removed one to 6 min after the termination of the injection, and the circulation to the clamped portion was reestablished. The abdominal wound was closed. The animals made a good recovery, and not a single one of them developed any sign of diabetes even temporarily. Ten or 14 days after the first operation the animals were relaparotomized or killed, and biopsies were taken from both the clamped and the untouched splenic portion of the pancreas. Sections were stained with chrome hematoxylin and phloxin,[3] and the

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* This work has been supported by grants from the Douglas Smith Foundation for Medical Research of the University of Chicago, and from Eli Lilly and Co., Indianapolis.

[1] Goldner, M. G., *Bull. N. Y. Acad. Med.*, 1945, **21**, 44.

[2] Leech, R. S., and Bailey, C. C., *J. Biol. Chem.*, in press.

[3] Gomori, G., *Am. J. Path.*, 1941, **17**, 395.

TABLE I.

Dog No.	Dose of alloxan mg/kg	Clamps removed after minutes	Changes	
			in clamped portion	in splenic portion
610	60	6	0	+
608	60	6	0	++
712	75	6	0	+++
714	75	5	0	0
715	75	5	0	++
716	75	3	0	0
411	75	3	0	0
409	75	2	0	+++
410	75	1	0	++
415	75	1	0	0
404	100	3	0	0
614	100	3	+	+++
405	100	2	0	++

0 = normal.

+ = questionable or slight changes (predominance of alpha cells in a number of islets).

++ = moderate damage (predominance of alpha cells in the majority of the islets; beta cells scanty).

+++ = severe damage (islets consist of alpha cells only; beta cells absent or only occasional specimens seen).

islets examined for evidences of alloxan damage. Table I. shows the results.

Table I. shows 2 interesting things: First, the unusually high percentage of animals insensitive to the doses of alloxan used in this experiment. Second, whenever changes developed, they were invariably far more severe in that portion of the gland the blood supply of which had not been interfered with. In only one animal did changes of questionable significance develop in the clamped portion of the gland, whereas in all other animals clamping for 1 to 6 min afforded full protection against alloxan damage.

In addition to the changes tabulated, in dogs No. 404 and 405 beta cells were found singly and in very small groups scattered throughout the acinar parenchyma. This very unusual finding may indicate a process of regeneration.

Summary. Clamping of a portion of the dog's pancreas during and for the period of 1 to 6 min after the injection of a diabetogenic dose of alloxan will protect that portion of the gland from alloxan damage while in the unclamped part the beta cells will degenerate. This observation shows that the toxic effect of alloxan is limited to a few minutes immediately following the injection.

14908 P

Certain Properties of a New Physiologically Absorbable Sponge.

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The clinical utility of thrombin for hemostasis is enhanced by its application in a porous matrix which may be left intact and will be physiologically absorbed. For this purpose certain products prepared from human fibrinogen,[1,2] oxidized cellulose,[3-5] and possibilities for a starch preparation,[6] have been reported. Each of these has sufficient limitations to encourage a preliminary report on a substance developed in this laboratory for use as a biologically absorbable sponge.

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[1] Ingraham, F. D., and Bailey, O. T., *J.A.M.A.*, 1944, **126**, 680.

A water-insoluble gelatin-base sponge has been obtained by foaming a specially prepared gelatin solution, which is then air dried in Monel metal forms and subsequently sterilized before biologic use.

The dried product is a light, off-white, nonelastic, tough, porous, mold that may be cut into any shape or size. There is no tendency to disintegrate even with relatively rough handling. A piece may be rapidly wet if worked vigorously in the damp fingers or sterile gloves to moisten the cell walls and expel air from the numerous pores; it will then engorge liquid readily and may be squeezed out and used repeatedly.

Physical properties of the material have been reasonably uniform among the several lots which have been studied. A 10 mm cube weighs aproximately 9 mg and will imbibe about 50 times its weight (450 mg) of water, or 45 times its weight (415 mg) of well-agitated, oxalated, whole bovine blood.

The sponge, a gelatin product, was unlikely to be antigenic. This possibility was, however, investigated. Two rabbits were subjected to a series of 5 injections with the bulk solution; two others with a saline extract of the finished product. These rabbits and 2 controls were bled on the 7th day following the last sensitization injection. Serums obtained were layered with dilutions of the antigens and observed for ring formation; later the tubes were agitated and followed for precipitate. All tubes remained clear. Precipitin assays repeated on the 11th and 15th days also were negative. From 3 albino rats, implanted in muscle with sponge 23 days earlier, serums were collected. These were titrated for precipitins, but again the evidence indicated the sponge to be nonantigenic.

Jenkins[7] has shown a relationship between enzymatic digestion of catgut *in vitro* and physiologic absorption *in vivo*.

By use of a standardized technique, which employed a solution of 1% U.S.P. XII pepsin, 100 mg pieces of sponge were digested in about 30 minutes; fibrin foam* yielded similar values.

A preliminary absorption study was made with 21 albino rats weighing approximately 300 g. Into a hind leg muscle of each was implanted a piece of sponge (6 × 8 × 8 mm) which was wet with a solution of bovine thrombin having a potency of 500 units† per cc. At intervals the animals were sacrificed and the implant areas visually examined. Only a small transparent film was in evidence on the 23rd day and no foreign material could be identified 30 days after implantation.

Further absorption data are now being accumulated from 28 albino rats which were implanted with materials in 4 different muscle areas of the two hind extremities. All implanted pieces measured, dry, 5 × 8 × 8 mm. Bovine thrombin solution as described previously was employed. In muscle region A was implanted a piece of fibrin foam soaked in thrombin solution; in region B, a piece of starch sponge‡ soaked in thrombin; in C, gelatin sponge soaked in thrombin; and in D, a piece of gelatin sponge soaked in a solution of penicillin which had a potency of 5,000 Oxford units per cc. Considerable difficulty was experienced in implanting the starch sponge, as in a wet condition it was friable and

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[5] Putnam, T. J., *Ann. Surg.*, 1943, **118**, 127.

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[7] Jenkins, H. P., and Hrdina, L. S., *Arch. Surg.*, 1942, **44**, 984.

* The fibrin foam utilized in this study was prepared by The Upjohn Company in collaboration with Dr. Edwin J. Cohn of the Department of Physical Chemistry, and the Plasma Fractionation Laboratory, Harvard Medical School, from blood collected by the American Red Cross, under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard University. The material used came from jars sorted out from a regular production lot because of minor imperfections.

† Clotting activity has been standardized in our laboratory such that if 0.1 cc of a solution clots 0.5 cc of specially oxalated bovine plasma in 15 seconds, that solution is considered to have a potency of 30 units per cc.

‡ Obtained through the courtesy of Dr. M. M. MacMasters, Northern Regional Research Laboratory, Peoria, Ill.

permitted very little manipulation.

Two animals have been sacrificed at regular intervals and specimens taken of each region, for gross inspection and histologic examination. No starch material could be visually identified after the 10th day. Absorption of the gelatin sponges was complete by the 30th day. After 50 days, small yellow nodules of fibrin foam were readily identified; their size indicated considerable but not complete absorption during this period of time. All operative regions appeared normal and well healed.

Details as to techniques, and histologic and experimental results on these and other studies of the gelatin sponge, including a series of neurosurgical investigations with 50 monkeys, are being completed.

The authors wish to express their appreciation to Dr. Glenn C. Bond for the immunologic assays, and to Mr. Stuart L. Long for his assistance in the laboratory and with the animals.

14909

Alleviation of Anaphylactic Shock in Guinea Pigs With Synthetic Benzhydryl Alkamine Ethers.

Earl R. Loew* and Margaret E. Kaiser.

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Recent studies in this laboratory have shown that synthetic benzhydryl alkamine ethers are capable of preventing fatal asthma induced in guinea pigs by administration of histamine either intravenously or by inhalation of atomized aqueous solutions.[1] The experimental data revealed that the potency of several benzhydryl ethers equalled or exceeded that demonstrated for two Fourneau histamine antagonists, 2-isopropyl-5-methyl-phenoxyethlydiethylamine (929F) and N-phenyl-N-ethyl-N′-diethylethylenediamine (1571F). Staub and Bovet[2-4] and others[5-13] have studied the antihistamine and antispasmodic properties of these Fourneau compounds.

Experiments have been conducted to determine whether benzhydryl ethers are effective in alleviating anaphylaxis in guinea pigs. β-Dimethylaminoethyl benzhydryl ether and β-morpholinoethyl benzhydryl ether, both as hydrochlorides, were selected for this study because of their effectiveness in alleviating histamine-induced asthma.[1]

Methods. Guinea pigs of both sexes weighing 250 to 350 g were passively sensitized by intraperitoneal injection of 0.5 cc of rabbit anti-ovalbumin serum. The rabbits had been

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* Present address: Department of Pharmacology, University of Illinois College of Medicine, 1853 West Polk Street, Chicago 12, Ill.

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[2] Bovet, D., and Staub, A. M., *Compt. rend. Soc. de biol.*, 1937, **124**, 547.

[3] Staub, A. M., and Bovet, D., *Compt. rend. Soc. de biol.*, 1937, **125**, 818.

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[11] Burchell, H. D., and Varco, R. L., *J. Pharm. and Exp. Therap.*, 1942, **75**, 1.

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TABLE I.
Efficacy of Benzhydryl Ethers in Decreasing Percentage Mortality in Guinea Pigs Subjected to Anaphylactic Shock.

Treatment	Dose mg/kg I.P.	Mortality			LD-50, I.P. in 100-125 g rats mg/kg	Degree of shock§					
		Ratio†	%	P‡		0§	*	**	***	****	F.S.
Untreated controls	—	23/45	51			2	13	6	1		23
Theophylline-ethylene-diamine (Aminophylline	50	1/9	11	<0.01	252		1	6		1	1
N-Phenyl-N-ethyl-N′-diethylethylene diamine hydrochloride (1571F)	3	2/13	15	<0.01	142	1	1	7	2		2
β-Dimethylamino-ethyl benzhydryl	3	1/14	7.1	<0.01	82		5	4	3	1	1
ether hydrochloride	12	1/15	6.6	<0.01		2	6	6			1
β-Morpholinoethyl	3	5/14	36	0.3			2	6	1		5
benzhydryl ether	6	6/15	40	0.3		2	3	2	1	1	6
hydrochloride	12	0/12	0	<0.01	185	1	3	7	1		

$$\dagger\ \text{Mortality ratio} = \frac{\text{No. died}}{\text{No. used}}$$

‡ P values taken from Fisher's table; value less than 0.05 indicates significant difference.

§ O, no symptoms.

* Teeth chattering, fur ruffled.

** Sneezing, dyspnea, excitation.

*** Urination, defecation, convulsions, gasping, cyanosis.

**** Severe shock, collapse, coma, gasping, cyanosis.

F.S., fatal shock.

immunized with crystalline ovalbumin[†] by injecting 1.0 cc of a 0.2% solution intravenously 3 times weekly for 3 or 4 weeks, at which time the antibody titer of the blood serum was considered to be sufficiently high as indicated by the fact that precipitins were demonstrated with serum dilutions of 1:100.

Twenty-four hours after passive sensitization of the guinea pigs anaphylactic shock was induced by a shocking dose of 2.0 cc of 0.2% aqueous solution of crystalline ovalbumin. The jugular vein was exposed under local anesthesia produced by subcutaneous infiltration of 1.0 cc of 1.0% procaine. Intrajugular injections of antigen were made into untreated control animals and into those which had received an intraperitoneal injection of a drug 15 min previously.

Results. The data (Table I) reveal that each of the chemical compounds in adequate dosage was effective in significantly reducing the incidence of fatal anaphylactic shock in sensitized guinea pigs. By comparison with the other compounds, the dose of aminophylline used to demonstrate anti-anaphylactic action was large but probably necessary in view of its low potency.[14, 15] β-Dimethylaminoethyl benzhydryl ether and 1571F were effective in reducing mortality when doses of 3.0 mg/kg were administered. Lower doses were not employed so the exact relative potency of these two compounds was not determined. β-Morpholinoethyl benzhydryl ether did not exhibit definite anti-anaphylactic activity with doses of 3.0 and 6.0 mg/kg but activity was demonstrated with a dose of 12.0 mg/kg. It is therefore concluded that β-dimethylaminoethyl benzhydryl ether is more than twice as active as β-morpholinoethyl benzhydryl ether in alleviating anaphylactic shock.

The acute toxicity (Table I) of β-morpholinoethyl benzhydryl ether is approximately one-half that of β-dimethylaminoethyl benzhydryl ether but because of the high potency of the latter compound its therapeutic index is greater.[1]

[†] Supplied through the kindness of Dr. Gertrude Rodney, Department of Immunochemistry, Parke, Davis and Company, Detroit, Mich.

[14] Young, R. H., and Gilbert, R. R., *J. Allergy*, 1941, **12**, 235.

[15] Emmelin, N., Kahlson, G. S., and Lindstrom, K., *Acta physiol. Scandinav.*, 1941, **3**, 39.

Although the incidence of mortality in guinea pigs subjected to anaphylactic shock was significantly reduced by each of the compounds a complete suppression of symptoms was not observed. In most instances, however, small doses of drugs were purposely employed in order to increase the possibility of obtaining mortality data which would permit more accurate determination of the relative potency of the compounds.

Discussion. The anti-anaphylactic efficacy of aminophylline demonstrated in these experiments corroborates findings reported by Young and Gilbert[14] who employed intravenous doses of 30 to 60 mg/kg to reduce the mortality in guinea pigs subjected to histamine shock or anaphylactic shock. Emmelin *et al.*[15] were cognizant of the fact that the antihistamine or anti-anaphylactic activity exhibited by aminophylline and other xanthines was much inferior to that described for synthetic histamine antagonists such as N-phenyl-N-ethyl-N′-diethylethylenediamine (1571F) and 2-isopropyl-5-methyl-phenoxyethyldiethylamine (929F). Our findings relative to the marked effectiveness of 1571F in alleviating anaphylactic shock are in agreement with published data.[4,8]

Of the two ethers studied, β-dimethylaminoethyl benzhydryl ether proved to be more than twice as active as β-morpholinoethyl benzhydryl ether in reducing mortality in guinea pigs subjected to anaphylactic shock. With the atomized histamine technique approximately the same relative degree of potency was demonstrated.[1] It was also shown that β-dimethylaminoethyl benzhydryl ether, in doses of 3.0 mg/kg intraperitoneally, was capable of protecting against the lethal effect of intravenous injections of histamine. It is evident, therefore, that benzhydryl ethers, in small doses, are capable of reducing mortality of guinea pigs either following administration of histamine intravenously and by inhalation as an atomized aqueous solution or following liberation of histamine from tissues during the antigen-antibody reaction such as occurs in anaphylaxis.[16]

We have reported (1) that β-dimethylaminoethyl benzhydryl ether is twice as effective as 1571F in alleviating bronchoconstriction induced in guinea pigs by inhalation of atomized histamine. The data regarding anti-anaphylactic activity of these two compounds do not permit a precise determination of relative potency, but it is apparent that they are probably the most potent compounds studied thus far.

In view of the fact that these synthetic benzhydryl ethers are effective in alleviating both histamine shock and anaphylactic shock the data constitute additional indirect evidence that the chief symptoms of anaphylaxis are referable to histamine.

Summary. The hydrochlorides of β-dimethylaminoethyl- and β-morpholinoethyl benzhydryl ether proved to be markedly effective in alleviating anaphylactic shock in guinea pigs. The potency of the former compound may equal or exceed that of 1571F, the most effective Fourneau histamine antagonist. These antispasmodic benzhydryl ethers should prove useful in studies dealing with the role of histamine in various physiological mechanisms and pathological conditions.

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14910

Antibacterial Properties of Some Positive Univalent Iodine Compounds.

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The antibacterial behavior of iodine and many of its compounds is well known. The present report represents the results of experiments on a type of compound not previously tested, which because of its chemical structure and properties, purports to be an efficient germicidal agent.

Carlsohn[1] first prepared salts which are derivatives of the very labile base IOH, stabilized by co-ordination of the iodine with pyridine. The compounds were prepared by the action of iodine on a silver or mercurous salt in the presence of pyridine or a non-aqueous solution of pyridine. The chemical reactions may be summarized by representative equations.

$$AgX^{*} + 2\ py^{*} + I_2 \xrightarrow{\text{CHC13}} IpyX + AgI$$

$$Hg_2X_2 + 6\ py + 3I_2 \xrightarrow[\text{solvent}]{\text{inert}} 2\ (HgI_2\ 2\ py) + 2IpyX$$

The iodine in these compounds acts chemically as a positive ion. Upon the electrolysis of these substances in pyridine, iodine is liberated at the cathode; hydrolysis or reduction yields free iodine.

Compounds in which the acid radicals were the succinate, benzoate, phthalate and p-nitrobenzoate were prepared and tested for their antibacterial activity. Since preliminary tests revealed that the p-nitrobenzoate compound was most stable, the data in this report is chiefly concerned with this compound. Evidence is presented to show that monopyridine iodine (I) p-nitrobenzoate is bactericidal against *Staphylococcus aureus*, *Eberthella typhosa* and *Clostridium tetani* and that the growth of *Aspergillus niger* is inhibited by this compound. Tests evaluating the p-nitrobenzoate as a skin bactericide are also described.

Experimental. Broth Inhibition Tests. One cc of a 10^{-2} dilution of a 24-hr broth culture of *Staphylococcus aureus* (F.D.A. 209) was added to 9 cc of phenol coefficient broth.[2] 0.1 g of the p-nitrobenzoate compound was placed in the tube and after shaking, 1 cc quantities were withdrawn at 2 min intervals and plated in phenol coefficient agar. A total of 10 samples were cultured. After incubation for 48 hr, no growth was observed on plates inoculated with the test culture exposed 4 min or longer to the germicide. The number of colonies developing in control plates exceeded 1000.

The above experiment was carried out with *Eberthella typhosa* (Hopkins) except that 0.05 g of compound and one loopful (4 mm) of a 24 hr culture was used. At the end of 4 min exposure, no bacterial growth occurred in the subcultures, while the number of control colonies was 1000+.

Agar and Serum Agar Inhibition Tests. The inhibitory action of monopyridine iodine (I) p-nitrobenzoate against various bacteria in nutrient agar and serum agar was determined as described by Ruehle and Brewer[2] with one slight modification. Ten per cent human serum was used in place of horse serum. The results of these tests are shown in Table I.

Bactericidal Tests In Vitro. From the zones of inhibition in the experiments described above (with the exception of those plates containing *Aspergillus niger*) a 4 mm loopful of

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[1] Carlsohn, H., *Über eine Neue Klasse von Verbindungen des Positiv Einwertigen Iods,* 1932, Verlag von S. Hirzel, Leipzig.

* X signifies a univalent acid radical; py refers to pyridine.

[2] Ruehle, G. L. A., and Brewer, C. M., Circular No. 198, 1931, United States Department of Agriculture. *United States Food and Drug Administration Methods of Testing Antiseptics and Disinfectants.*

TABLE I.
Agar and Serum Agar Inhibition Tests.

	Zone of inhibition in cm†				
Organism	Serum agar 24	24	72	96	168 hr
S. aureus (F.D.A. 209)	0.9	1.2	1.5	—	—
E. typhosa (Hopkins)	1.3	1.6	1.7	—	—
Cl. tetani (American type culture)	—	—	—	1.9	—
A. niger	—	—	—	—	1.6

† The data given are the averages of 5 plates.

TABLE II.
Monopyridine Iodine (I) p-Nitrobenzoate-Bactericidal Tests *in vivo*.

	No. of mice	No. dead within 48 hr
Surgical controls	5‡	0
Hemolytic streptococci	10	10
Compound and streptococci	10‡	2
Compound alone	10‡	0

‡ Experiment observed for 16 days. No deaths occurred after 48 hours.

agar was withdrawn, mashed and placed into 10 cc of phenol coefficient broth and incubated for 5 days. In the case of *Clostridium tetani* the phenol coefficient broth contained glucose. In each instance, the absence of growth after 5 days incubation showed that monopyridine iodine (I) p-nitrobenzoate is bactericidal towards the organisms named.

Bactericidal Tests In Vivo. These experiments were performed according to the method of Sarber.[3] Pieces of abdominal skin coated respectively with *Hemolytic streptococci* (strain 1048M) alone, the compound alone, and streptococci plus compound were sealed into the peritoneum of mice and the animals were observed for a number of days. The results are tabulated in Table II.

Experiments on Hydrolysis Products. When compounds of the type IpyX hydrolyze, pyridinium iodate and the pyridinium salt of the acid radical are among the products formed. To determine whether these products of hydrolysis might contribute to the germicidal action, solutions of pyridinium iodate and pyridinium phthalate were tested against *Staphylococcus aureus* (F.D.A. 209) in 24 hr broth cultures. Since the bactericidal action of these compounds was negligible at the end of 30 min, it was concluded that these substances made little or no contribution to the antibacterial behavior of the original positive univalent iodine compounds.

Value as Skin Antiseptic. To test the local toxicity of one application of the p-nitrobenzoate, the following experiment was performed. Ten guinea pigs were anesthetized and their abdomens were shaven clean, washed, and dried. Two 1 cm square areas were scraped on each side of the abdomen until a serous fluid issued from the wounds. Into one area p-nitrobenzoate was rubbed, while saline solution was placed on the control area. The guinea pigs were examined each day and the length of time for the test areas and controls to heal was recorded. The time of healing for the wounds containing the compound on 9 of the animals was identical with that required by the control area. On none of the animals did the skin show signs of necrosis.

In order to evaluate the ability of monopyridine iodine (I) p-nitrobenzoate to act as a cutaneous germicide, tests were performed as described by Novak and Hall.[4] An area on the flexor side of the forearm of a human subject was first swabbed with sterile saline and then a fine coating of powdered p-nitrobenzoate was applied in a similar manner. After 30 sec, a sterile watch glass filled with blood agar was inverted over the area and held there 5 min, after which it was removed and incubated. After 48 hr, the colonies on the impression plates were counted. In all cases where no growth occurred, *Staphylococcus aureus* (F.D.A. 209) was streaked over the blood agar and after 24 hr incubation, the plates were examined again. In each instance growth occurred, indicating that an insufficient amount of the compound was removed from the skin surface to act as a bacteriostatic agent in the blood agar medium. The flexor surface of the other arm was used as a control. Data on 15 subjects showed the compound to be destructive to approximately 94% of the bacteria on the skin. No signs of local irritation were observed in these subjects.

Summary. From these experiments, one can conclude that monopyridine iodine (I) p-nitrobenzoate rapidly destroys common pathogenic bacteria and that it is relatively non-toxic and non-irritating.

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[3] Sarber, R. W., *J. Bact.*, 1942, **43**, 50.

[4] Novak, M., and Hall, H., *Surgery*, 1939, **5**, 560.

14911

Phenomenon of Precipitation on Mixing Hemoglobin With Tissue Extract Antigen.*

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It was observed in this laboratory that precipitation occurs on mixing laked erythrocytes, referred to as hemoglobin solution, with tissue extract (Kahn) antigen. It was also observed that hemoglobin solutions derived from malarial (vivax) and nonmalarial erythrocytes showed some differences in their zones of precipitation. To our knowledge such observations have not heretofore been reported.

Zones of Precipitation with Malarial and Nonmalarial Hemoglobin Solutions: Hemoglobin solution is prepared as follows: Clots from whole blood specimens are broken up mechanically in about 5 cc of physiologic salt solution. Unbroken clumps remaining are removed by filtration through gauze. The resulting red cell suspension is washed free from serum as in the preparation of sheep cells for complement fixation tests. Three to four washings with saline are usually sufficient. The washed cells are packed in a calibrated centrifuge tube by centrifugation for 15 min at 1,800 rpm, the volume of the packed cells recorded, and the water-clear supernatant fluid discarded. The tubes are allowed to stand for 10 min and the residual supernatant fluid is removed as completely as possible. Distilled water is added to the packed cells to make a 1:5 dilution and the cells are hemolyzed as thoroughly as possible by stirring intermittently with a wooden applicator during a 10 min period. The resulting turbid solution is centrifuged for 15 min at 1,800 rpm. The supernatant hemoglobin solution is removed carefully from the sediment with a bulb pipette and is employed in preparing serial dilutions with distilled water ranging from 1:5 to 1:1,920.

Antigen suspension is prepared by mixing standard Kahn antigen with 0.3% NaCl solution instead of 0.9%. The method of mixing and the aging of the suspension before use are the same as employed in the Kahn test.

The serial dilutions of hemoglobin solution are added to the antigen suspension in the following ratios: 1:4, 1:2, 1:1, 3:1, 6:1, 12:1, 18:1, 24:1 and 30:1 (Table I.). The tubes are shaken by hand for a few seconds to assure proper mixing and the results are read.

Two main types of precipitation zones were obtained, the "limited zone" and the "wider zone," illustrated in Table I. It is apparent from this table that the two types differ mainly in the precipitation results obtained with 1:15 and 1:30 dilutions of hemoglobin in the 6:1 through the 24:1 ratios. In this "differential range," positive precipitation results are noted in the "wider zone" and negative results in the "limited zone."

Both types of precipitation zones were obtained with hemoglobin solutions of malarial and nonmalarial origin, but the hemoglobin solutions of malarial origin tended to give more frequently the "wider zone" of precipitation. The malarial specimens were provided us through the courtesy of the U. S. Naval Medical School, Bethesda, Maryland, and of the U. S. Naval Hospital, San Diego, California. The nonmalarial specimens were obtained from the Serological Laboratory of the University of Michigan Hospital.

Precipitation in the "Differential Range" with Malarial and Nonmalarial Hemoglobin Solutions: Table II. presents precipitation results of hemoglobin solutions from 10 malarial and 10 nonmalarial blood specimens.

These solutions in each instance were diluted 1:15 and 1:30 with distilled water and were tested with antigen suspension in ratios of 6:1 through 24:1. These ratios and hemoglobin dilutions represent the differential range of

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* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Regents of the University of Michigan.

TABLE I.
Illustration of Zones of Precipitation Shown by Hemoglobin with Tissue Extract Antigen.

Hemoglobin solution, cc	0.05	0.05	0.1	0.15	0.15	0.15	0.18	0.24	0.30
Antigen suspension, cc	0.2	0.1	0.1	0.05	0.025	0.0125	0.01	0.01	0.01
Ratios of hemoglobin dilution to antigen suspension	1:4	1:2	1:1	3:1	6:1	12:1	18:1	24:1	30:1
Dil. of hemoglobin with distilled water	Limited zone of precipitation								
1:5	4*	4	—	—	—	—	—	—	—
1:10	4	4	4	2	—	—	—	—	—
1:15	4	4	4	4	—	—	—	—	—
1:30	4	4	4	4	2	—	—	—	—
1:60	4	4	4	4	4	4	4	—	—
1:120	—	3	4	4	4	4	4	2	1
1:240	—	2	4	4	4	4	4	4	—
1:480	—	—	—	4	4	4	4	3	—
1:960	—	—	—	—	—	4	4	—	—
1:1920	—	—	—	—	—	—	—	—	—
	Wider zone of precipitation								
1:5	4	4	4	4	—	—	—	—	—
1:10	4	4	4	4	4	2	—	—	—
1:15	4	4	4	4	4	4	4	3	—
1:30	4	4	4	4	4	4	4	4	1
1:60	4	4	4	4	4	4	4	4	1
1:120	—	—	4	4	4	4	4	4	4
1:240	—	—	2	4	4	4	4	4	2
1:480	—	—	—	2	4	4	4	2	1
1:960	—	—	—	—	—	4	4	—	—
1:1920	—	—	—	—	—	—	—	—	—

* The numbers 4, 3, 2 and 1 represent precipitation in decreasing scale.

precipitation between hemoglobin solutions of malarial and nonmalarial origin. The blood specimens in this experiment were aged for 24 hr at room temperature. Ageing of blood specimens was found to be an important step in obtaining precipitation in the differential range. It is evident from the table that the hemoglobin solutions of malarial blood specimens gave more marked precipitation reactions than those of nonmalarial blood specimens.

A more extensive precipitation study was undertaken with 137 malarial blood specimens which had been received through the mails and had undergone approximately 48 hr ageing. About the same number of nonmalarial specimens, similarly aged, were employed as con-

TABLE II.
Precipitation with Malarial and Nonmalarial Hemoglobin Solutions.

	Malarial							Nonmalarial					
	Hemoglobin dilution with distilled water												
	1:15			1:30				1:15			1:30		
	Ratios of hemoglobin dilution to antigen suspension												
Spec. No.	6:1	12:1	18:1	12:1	18:1	24:1	Spec. No.	6:1	12:1	18:1	12:1	18:1	24:1
1	—	—	—	4	4	3	1	—	—	—	—	—	—
2	—	—	—	4	4	4	2	—	—	—	4	—	—
3	—	—	—	2	—	—	3	—	—	—	2	2	1
4	—	—	—	4	4	4	4	—	—	—	—	—	—
5	4	4	3	4	4	4	5	3	1	—	4	4	4
6	4	—	—	4	4	4	6	—	—	—	—	—	—
7	—	—	—	4	2	—	7	—	—	—	—	—	—
8	—	—	—	1	—	—	8	—	—	—	—	—	—
9	—	—	—	—	—	—	9	—	—	—	1	—	—
10	—	—	—	2	1	—	10	—	—	—	3	2	2

TABLE III.
Precipitation with Malarial and Nonmalarial Hemoglobin Solutions (Employing Aged Blood Specimens).

Malarial Spec. 137		Nonmalarial Spec. 138		Nonmalarial Spec. 306 Aged 4 hr at room temp.	
Aged about 48 hr at room temp.					
No.	%	No.	%	No.	%
Precipitation in the 1:15 and 1:30 hemoglobin dilutions*					
125	91	31	24	7	2
Precipitation only in the 1:30 hemoglobin dilution					
9	7	67	28	17	4
No precipitation in the 1:15 and 1:30 hemoglobin dilutions					
3	2	30	48	282	94

* The 1:15 hemoglobin dilution was examined in ratios of 6:1, 12:1 and 18:1 with antigen suspension. The 1:30 hemoglobin dilution was examined in ratios of 12:1, 18:1 and 24:1 with antigen suspension. If precipitation occurred in one or more ratios of either dilution, the result was recorded as positive.

trols. Table III. summarizes the results obtained.

As indicated in the table, 91% of malarial hemoglobin solutions in 1:15 and 1:30 dilutions with distilled water gave positive precipitation reactions. In addition, 7% of the hemoglobin solutions gave precipitation only in the 1:30 dilution. The remaining 2% showed no precipitation. Of the nonmalarial hemoglobin solutions, 24% gave precipitation both in the 1:15 and 1:30 dilutions and 28% only in the 1:30 dilution. The remaining 48% showed no precipitation. It is thus evident that under the ageing conditions specified, the malarial hemoglobin solutions showed a greater tendency toward precipitation than the nonmalarial hemoglobin solutions.

In Table III. is also presented an experiment in which 306 nonmalarial blood specimens were aged only 4 hr at room temperature. When hemoglobin dilutions from these specimens were examined with antigen suspension, only 6% showed precipitation. Preliminary findings indicate that hemoglobin dilutions from fresh malarial blood specimens give similar precipitation results.

Studies are in progress to determine the nature of the precipitation phenomenon described in this report and if the phenomenon is applicable to the development of a practical method for differentiation between hemoglobin solutions of malarial and nonmalarial origin.

Summary. It was observed that hemoglobin solutions (laked human erythrocytes in distilled water) when mixed with tissue extract antigen (Kahn antigen suspension) under proper conditions show immediate precipitation. This phenomenon of precipitation is manifested in a relatively wide range of hemoglobin-distilled water dilutions and also in various ratios of these dilutions to antigen suspension. Hemoglobin solutions derived from malarial blood specimens show a tendency toward a wider range of precipitation than hemoglobin solutions derived from nonmalarial blood specimens. Ageing of the blood specimens at a temperature above refrigeration, such as room temperature, is an important factor in increasing the tendency toward such precipitation.

14912

Release of Curare-Like Agent from Healthy Muscle and Its Bearing on Myasthenia Gravis.*

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Several investigators, notably Nevins,[1, 2] have suggested the possibility of the presence in the blood or tissues of patients with myasthenia gravis of an abnormal substance or metabolite with a curare-like action which raises the threshold of skeletal muscle to the effects of acetylcholine. Walker[3] tested this

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TABLE I.
Effect of serum of cat on the neuro-muscular transmission of frog.

Time of stimulation after inj. of serum in min.	Magnitude of contraction of the gastrocnemius muscle of the frog in % of control during electrical stimulation of the sciatic nerve followed by the S.E. of the mean. Serum collected from:					
	immobilized limb	intermittently stimulated limb	continuously stimulated limb	passively moved limb	control Ringer's sol.	frog with excised heart
½	100±1.0	99±0.9	99±0.3	98±0.6	99±0.2	99±0.4
1	100±0.7	99±0.3	97±0.2	94±1.0	99±0.3	98±0.8
1½	100±0.8	99±0.5	96±0.4	92±1.8	100±0.4	97±0.8
2	99±0.9	98±1.2	94±0.3	90±1.7	98±0.5	98±0.9
2½	98±1.1	97±0.5	93±0.9	88±1.9	100±0.2	98±1.3
3	99±0.9	97±0.6	92±0.7	83±1.9	100±0.4	98±0.9
3½	98±0.5	97±0.3	91±1.1	85±1.8	101±0.3	99±0.6
4	98±0.6	98±0.7	90±0.5	83±2.0	100±0.7	99±0.5
4½	99±0.5	97±0.4	89±0.8	81±1.7	102±0.2	99±0.7
5	98±0.4	99±0.8	88±0.7	79±2.0	100±0.2	99±0.3
5½	98±0.7	99±0.5	88±0.8	76±1.9	101±0.4	99±0.5
6	99±0.2	98±0.2	87±0.9	74±1.6	102±0.2	98±0.2
6½	98±0.4	98±0.7	86±0.9	74±2.2	100±0.3	97±0.6
7	99±0.6	98±0.6	83±1.0	73±2.5	98±0.5	98±0.7
7½	98±0.5	98±0.5	82±0.7	69±2.6	99±0.3	98±0.5
8	99±0.4	98±0.4	82±1.3	69±2.3	100±0.7	99±0.6
8½	98±0.7	97±0.8	81±0.9	67±2.1	98±0.8	100±1.1
9	100±0.4	97±0.7	80±1.1	66±2.4	102±0.2	99±0.7
9½	99±0.5	96±0.7	78±1.4	65±2.6	100±0.4	99±0.3
10	97±0.4	96±1.2	78±1.3	62±2.5	100±0.4	100±0.9
20 min. rest period.						
30½	110±0.3	109±1.1	110±0.6	108±0.6	107±0.5	108±0.9
31	112±0.8	109±0.6	111±0.3	109±0.3	109±0.8	109±1.1
31½	112±0.7	110±0.5	111±0.5	109±0.7	110±0.2	109±0.5
32	110±0.4	109±0.9	111±0.7	110±0.4	109±0.4	107±0.7
32½	110±0.7	111±0.8	111±0.3	111±0.7	108±0.4	110±0.8
33	109±0.3	109±1.2	110±0.5	112±0.5	108±0.4	110±0.5
No. of exp.	28	15	13	23	60	10

theory by occluding the circulation in the upper limbs of patients with myasthenia gravis and exercising the muscles of the forearm. On releasing the constriction an increase in the clinical signs was observed. She concluded that muscles of patients with myasthenia gravis liberate a chemical substance which passes into the circulation and produces a neuro-muscular block in the motor end-plates of skeletal muscle. Wilson and Stoner[4] found that the serum of patients with myasthenia gravis not under prostigmine treatment collected after voluntary muscle work, when tested on the isolated nerve-muscle preparation of the frog, produces a block in neuro-muscular transmission. This effect is not obtained with serum collected from healthy subjects or patients during prostigmine treatment. These authors conclude that in contrast to the healthy subjects the blood of patients with myasthenia gravis contains a substance with curare-like action which interferes with neuro-muscular transmission.

It occurred to us that the depression of the neuro-muscular transmission in the experiments of Walker[3] and Wilson and Stoner[4] may be explained otherwise. It is likely that a dynamic equilibrium exists between stimulator substances (acetylcholine-like substance liberated at the synapse,[5-9] decomposition

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[1] Nevin, S., *Brain*, 1934, **57**, 239.

[2] Nevin, S., *J. Neurol. and Psychiatry*, 1938, **1**, 120.

[3] Walker, M. B., *Proc. Roy. Soc. Med.*, Series B., 1938, **31**, 722.

[4] Wilson, A., and Stoner, H. B., *Quarterly J. Med.*, 1944, **37**, 1.

[5] Bacq, Z. M., *Ergebnisse Physiol.*, 1935, **37**, 82.

[6] Eccles, J. C., *Ergebnisse Physiol.*, 1936, **38**, 339.

[7] Brown, G. L., *Physiol. Rev.*, 1937, **17**, 485.

[8] Dale, H., *Harvey Lectures*, 1937, **32**, 229.

products thereof, some metabolites) and substances with curare-like action (other metabolites) during and after muscle contraction of healthy subjects. It is likely that agents with curare-like action are present after muscle contraction since fatigued muscles of healthy subjects behave as slightly curarized muscles as far as summation of stimuli,[10–12] post-tetanic potentiation of single twitches,[13] and response to tetanizing currents[13–15] are concerned.

Should the dynamic equilibrium be disturbed by an unusual amount of the normally occurring agent, with curare-like effect, by some unusual curare-like agent, or by a decrease of the stimulating agent present the curare-like effect appears. In the experiments of Wilson and Stoner[4] with patients not treated with prostigmine the curare-like effect could have been due to the insufficient amount of stimulator substances released into the blood during muscle work. The effect of serum of patients with myasthenia gravis observed on the neuro-muscular transmission of the frog might have been due to the lack of stimulator substances in the serum since administration of prostigmine alone was sufficient to counteract the effect of the curare-like agent. This would seem to be so unless one postulates that physostigmine has other anti-curare effects than that exerted by the decrease of the decomposition of acetylcholine and increase of the potassium content of muscle.[16, 17]

The assumption of the appearance of the curare-like effect caused by a lack of sufficient amounts of potentiator substances would be substantiated if an agent with curare-like effect could be demonstrated in the serum of healthy subjects after muscle movements.

Method. Cats of 2-3 kg body weight were anesthetized with a Dial-Urethane solution (Ciba). After 2 hr 3 types of experiments were performed: (1) the femoral vein was ligated and the leg passively extended and flexed once per second for 10 min after section of the sciatic and femoral nerves; (2) the femoral vein was ligated and the sciatic nerve stimulated with an induction coil for 10 min either continuously, using a current strong enough to induce toe extension, or intermittently, alternating one second of stimulation with one second rest period using a weaker current but strong enough to induce slight muscle contraction; (3) the femoral vein was ligated for 10 min and the leg immobilized. After 10 min 3 cc blood was collected from the ligated vein, centrifuged, and the effect of serum on the neuro-muscular transmission of the frog was tested immediately.

The frog was prepared as follows: frogs of 18-23 g body weight were pithed and pinned to a board. A knee was fixed and the tendon of the gastrocnemius muscle attached to an isotonic lever. The movements of the muscle were registered by a kymograph. Electrodes were placed on the intra-abdominal branches of the sciatic nerve and the nerve was stimulated with an induction coil for 3 sec every 30 sec. After a steady height of contraction of the gastrocnemius muscle was reached, 0.2 cc serum of experimental cats were injected into the heart. Not more than 5 to 8 min elapsed between collection of blood from the cats and injection of the serum into the frog. The intermittent stimulation of the sciatic nerve of the frog was continued for 10 min as described previously and after a pause of 20 min the intermittent stimulation was resumed for 3 min. The muscle contraction in frogs injected with 0.2 cc mammalian Ringer's solution served as control.

Upon conclusion of the experiments the frogs were injected with Berlin blue to ascertain whether the blood supply of the gastrocnemius muscle remained adequate during the experiments.

Computation. The height of the tracings was measured. For each experiment 2 to 3

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[9] Schriever, H., *Ergebnisse Physiol.*, 1936, **38**, 877.

[10] Bremer, F., *Comp. rend. soc. biol.*, 1927, **97**, 1179.

[11] Bremer, F., *Comp. rend. soc. biol.*, 1929, **102**, 332.

[12] Bremer, F., *Am. J. Physiol.*, 1929, **90**, 297.

[13] Rosenblueth, A., and Morrison, R. S., *Am. J. Physiol.*, 1937, **119**, 236.

[14] Hofmann, F. B., *Pfluger's Arch.*, 1903, **95**.

[15] Bouman, H. D., *Arch. Neerl. Physiol.*, 1932, **17**, 307.

[16] Thompson, V., and Tice, A., *J. Pharmacol. Exp. Therap.*, 1941, **73**, 455.

[17] Wilson, A., and Wright, S., *Quart. J. Exp. Physiol.*, 1936, **26**, 128.

frogs were injected with Ringer's solution, 2 with the serum from the immobilized leg with occluded circulation and 2 with the moved or stimulated leg. In each tracing the contraction before injection served as 100% and each following value obtained was expressed as a % of this contraction.

Results. The height of contraction of the gastrocnemius muscle of the frog during the various experimental procedures is given in Table I. Serum collected from immobilized legs with occluded circulation did not modify the contraction of the gastrocnemius muscle of the frog. Serum collected from the limbs with occluded vein after intermittent stimulation of the sciatic nerve did not modify the contraction of the gastrocnemius muscle. Serum collected after 10 min of tetanus depressed the contraction of the gastrocnemius muscle (at the end of the 10 min test period the contraction was on the average of 19% below the values of the control) and the contraction of the gastrocnemius muscle returned to its original state during the 20 min of rest. Serum collected from passively moved legs with sectioned nerves and occluded femoral vein depressed the contraction of the gastrocnemius muscle. The contraction was on the average 35% less than the control at the end of the 10 min test period. A period of 10 min was chosen for convenience. It is a period long enough to show the initiation of the depression of the muscle contraction by the serum of the cat. The depression was greater when the period of stimulation of the frog preparation was prolonged. The muscle contraction returned to its original state during the 20 min rest period and behaved similarly to the control frogs thereafter. In these experiments the liberation of stimulator substances from the nerve endings of cat may not be entirely prevented, since, during the passive movements, the motor nerve may have been stimulated to some extent.

The injection of 0.2 cc serum induced an increase of the volume of blood of the frog, a temporary injury to the heart, and, if sera from passively moved limbs and continuously stimulated limbs were used, the cat serum induced a short block of the frog heart followed by a prolonged increase of the heart rate. To ascertain whether the changes of blood supply of the gastrocnemius muscle during and after the injection of the cat serum contributed to the decrease of the contraction of gastrocnemius muscle, experiments were performed using frogs with excised hearts. The height of contraction of the gastrocnemius muscle after stimulation of the sciatic nerve was very similar to that found in control frogs with intact hearts.

For purpose of further control the gastrocnemius muscle was directly stimulated through electrodes inserted into the tendons of the muscle. Since the extent of contraction was not significantly modified by the injection of cat sera into the frog it may be assumed that the decrease of the contraction of the gastrocnemius muscle during the stimulation of the sciatic nerve was due to the decrease of the neuro-muscular transmission.

Discussion. The above experiments suggest that during movements substances that depress the neuro-muscular transmission are passed into the blood. It may be asumed that in healthy animals the effect of these substances is counteracted by the presence of stimulator substances released at the synapse during the stimulation of the motor nerve since serum collected after intermittent stimulation of the motor nerve did not modify the neuro-muscular transmission of frog.

It is likely that the passive movements of the leg resulted only in stretching the muscles without inducing any contraction. It has been shown, however, that stretching increases the metabolism, oxygen consumption, and heat production of muscle. Just as in the case of muscle contraction, these processes depend perhaps on the organic phosphate content of the muscle, since the metabolic effects of stretching are not modified by poisoning the muscles with monoiodoacetic acid.[18, 19] Stretching induces pH changes similar to those induced by contraction.[20] Since continuous stimulation of the sciatic nerve for 10 min induced the release of some substances that depressed the neuro-muscular transmission of the frog it is possible that the substances released

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[18] Feng, T. P., *J. Physiol.*, 1932, **74**, 441.

[19] Feng, T. P., *J. Physiol.*, 1932, **74**, 455.

[20] Margaria, R., *J. Physiol.*, 1934, **82**, 496.

during the passive movements are also released during active movements.

The possibility that blood and tissues of patients with myasthenia gravis contain an abnormal substance or metabolite with curare-like effect cannot be excluded at present. However, the above experiments support the postulate that the symptoms of patients with myasthenia gravis and the demonstrated decrease in synthesis of acetylcholine in the presence of the serum[21, 22] and spinal fluid[23] of these patients are intimately related.

During prolonged stimulation of the preganglionic fibres of sympathetic ganglia acetylcholine synthesis takes place since the total amount of acetylcholine liberated may be several times that obtained from an unstimulated ganglion by extraction.[24, 25] If a similar synthesis occurs at the neuro-muscular synapse it is conceivable that decreased acetylcholine synthesis may lead to a decreased release of acetylcholine-like substances at the synapse during performance of work. It may be assumed that in patients with myasthenia gravis the decreased synthesis of acetylcholine leads to a disturbed dynamic equilibrium during performance of work resulting in a dominance of the agent with curare-like effect. The decreased liberation of acetylcholine-like substance at the synapse by itself, the dominance of the agents with apparent curare-like effect, and perhaps also other not yet identified processes manifest themselves clinically in the easy fatiguability of patients with myasthenia gravis.

Summary and Conclusions. (1) The effect of serum collected after passive movements of limbs of cats with occluded circulation on the neuro-muscular transmission of the frog was compared to the effect of serum obtained from immobilized limbs with occluded circulation, and from limbs with occluded circulation after intermittent or continuous electrical stimulation of the motor nerve. (2) Serum collected from passively moved limbs depressed the neuro-muscular transmission of the frog, whereas serum collected from immobilized limbs and limbs after intermittent stimulation of the motor nerve did not modify the neuro-muscular transmission. Serum collected from limbs after continuous stimulation of the motor nerve depressed the neuro-muscular transmission of the frog to a lesser extent. (3) The following postulate is offered: (a) that during performance of muscle work agents with curare-like effect are passed into the blood; (b) that in healthy subjects the effect of these substances is counteracted by sufficient amounts of stimulator substances or decomposition products thereof; (c) that in patients with myasthenia gravis the decrease of the synthesis of acetylcholine-like substances may manifest itself by the decrease of stimulator substances during the performance of work; and (d) that the lack of stimulator substances allows the usual agents with curare-like effect to manifest their presence during performance of work. The decreased release of acetylcholine-like substances at the synapse by itself, the dominance of substances with apparent curare-like effect, and perhaps also other not yet identified processes may lead to the easy fatiguability observed in patients with myasthenia gravis.

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[21] Torda, C., and Wolff, H. G., *Science*, 1943, **98**, 224.

[22] Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, **23**, 649.

[23] Torda, C., and Wolff, H. G., *Science*, 1944, **100**, 200.

[24] Brown, G. L., and Feldberg, W., *J. Physiol.*, 1936, **88**, 265.

[25] Kahlson, G., and MacIntosh, F. C., *J. Physiol.*, 1939, **96**, 277.

14913

A Tail Plethysmograph for Measuring Arterial Blood Pressure in Dogs.

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A successful and satisfactory method of determining the blood pressure without cannulating, puncturing or exteriorizing an artery has not been available for use on dogs. Because hematomas frequently developed after the blood pressure of hypertensive dogs had been taken by the method of Hamilton, we were stimulated to explore the possibilities of measuring the blood pressure of dogs indirectly by means of volume changes in the tail.

The tail plethysmograph developed for use on the rat by Byrom and Wilson,[1] and subsequently modified by Williams, Harrison and Grollman,[2] Friedman,[3] and Kempf and Page[4] was adapted for use on the dog. In suiting this apparatus to the dog it was necessary to compensate for certain differences between the tails of rats and dogs. The tails of rats are quite uniform in shape whereas those of dogs vary greatly in length, conformation at the base and degree of tapering from base to tip. Consequently it was not merely a matter of enlarging the apparatus used for the rat to the size necessary for the tails of dogs, but redesigning of the apparatus was necessary. Instead of a rubber-lined glass cuff as used on the tail of the rat, a latex bag (3 by 20 cm) with a cloth cover was wrapped around the base of the tail as is done with the arm in clinical sphygmomanometry. The cuff is likewise connected to a mercury manometer.

To accommodate tails of different lengths, a series of glass chambers or plethysmographs 18 to 22 cm long fashioned from glass tubing 22 mm in diameter was prepared. A 2 mm lipping of the proximal end of the plethysmograph allows the anchoring of a latex sleeve which when fitted to the tail prevents escape of water from the plethysmograph. The sleeves are easily made by dipping standard 15 cc centrifuge tubes in liquid latex a sufficient number of times to give the desired thickness.

At the closed distal end of the plethysmograph above and below are two offset outlets. To the one above is attached the water manometer, which is a 2 to 3 mm L shaped glass tube about 20 cm long. In order to reduce the inertia of the column of water the long arm of the L is placed horizontal to the long axis of the plethysmograph. The capacity of the plethysmograph is about 50 cc. By suspending the apparatus from an adjustable ringstand by a rubber sling the fluctuations of the water manometer caused by respiratory and other movements of the animal are greatly reduced (Fig. 1.).

The tail is prepared by clipping and shaving to allow a more effective seal at the proximal end. To prevent the skin of the tail from becoming watersoaked, which diminishes the sensitivity of the readings, the tail was rubbed with petrolatum. To aid further in this respect the tail was kept in the apparatus only when necessary for taking readings. Care was exercised to obtain the proper placement of the tail in the apparatus. The lip of the plethysmograph should engage the tail with a minimum of pressure so as to avoid venous stasis. Shaving soap lather or brushless shaving cream aids in making the latex sleeve into which the tail is inserted watertight.

Like the plethysmograph, the blood pressure cuff is suspended by a rubber sling. Best results were obtained by applying the cuff each time a series of readings was taken. The proximity of the cuff with reference to the chamber appeared to be inconsequential but the cuff should be wide enough to avoid a

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[1] Byrom, F. B., and Wilson, C., *J. Physiol.*, 1938, **93**, 301.

[2] Williams, J. R., Jr., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

[3] Friedman, S. M., *J. Lab. and Clin. Med.*, 1941, **27**, 240.

[4] Kempf, G. F., and Page, I. H., *J. Lab. and Clin. Med.*, 1942, **27**, 1192.

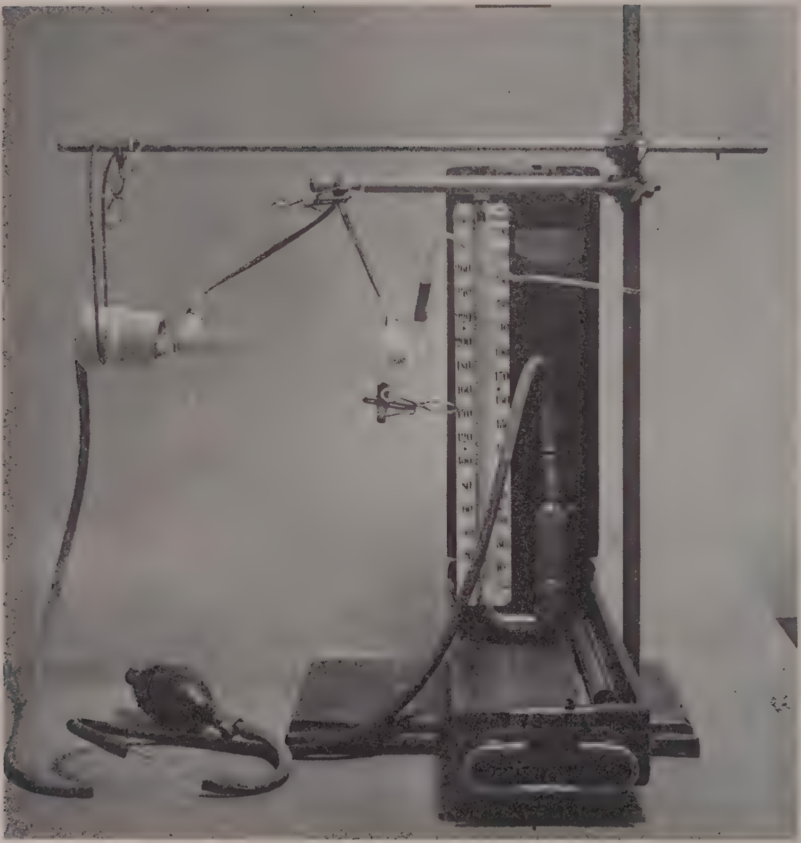

FIG. 1.
The tail plethysmograph.

narrow band of occlusion on inflation.

To measure the blood pressure, the chamber is slowly filled with water at room temperature or above and all bubbles of air are excluded. Water is forced up into the manometer tube for a distance of about 5 cm. There is at once a fluctuation of the water level of 4 to 6 mm due to the pulse. The tail cuff is then rapidly inflated to a level about 20 mm of mercury above an occlusive pressure. The cuff is then slowly deflated. If the sphygmomanometer scale and the water manometer are properly aligned, both can be observed simultaneously. At the first sign of a pulsating rise of the water in the manometer the level of the mercury is noted and this reading is taken as the systolic blood pressure. A satisfactory index of diastolic blood pressure was not found. Friedman's end point, the change of a pulsating rise in the water manometer to a slower steady rise, is not applicable to the dog since a marked pulsation is present even with no inflation of the cuff. After an observation of the systolic blood pressure the cuff should be rapidly deflated and a rest of 5 to 15 seconds given before repeating the procedure. The most satisfactory results were obtained when 4 or 5 readings were taken in rapid succession and the chamber was emptied until the next observation. If considerable time was to intervene, the tail was removed from the apparatus and again replaced just before the next observations were made.

Results. The apparatus gave constant and accurate values of blood pressure in anesthetized (pentobarbital sodium, 25 mg per kg)

TABLE I.
Data on Blood Pressures of Anesthetized, Trained and Untrained Dogs, Obtained by Direct (Arterial Puncture) and Indirect (Tail Plethysmograph) Means.

			Blood pressure, mm of mercury		
Dog	Anesthesia	Trained	Plethysmograph	Femoral artery	Difference
1	+	+	140	135	5
	—	+	140	130	10
	—	+	146	136	10
	+	+	132	126	6
	+	+	132	126	6
2	+	—	175	168	7
3	+	—	170	162	8
	+	—	138	132	6
	—	—	175	132	43
4	—	—	185	145	40
	+	—	142	134	8
5	+	—	118	110	8
	+	—	146	140	6
	+	—	118	112	6

and in well-trained dogs. It was found that inflation of the cuff sometimes caused certain dogs to contract the caudal muscles enough to give blood pressure values that were too high. In such animals local caudal or spinal block prevented this from occurring. The data obtained with the tail plethysmograph were simultaneously checked against values obtained with pressures taken by direct arterial puncture and mercury manometer. Representative data on the blood pressure obtained from trained and untrained dogs, with and without anesthesia, are given in Table I. The blood pressure of the anesthetized dog taken by the plethysmograph agreed very well with that obtained by direct arterial puncture but was, on an average, 6 to 8 mm of mercury higher, while in the unanesthetized trained dog the blood pressure was about 10 mm higher. In the unanesthetized untrained animal differences of as much as 40 mm of mercury were observed. One must, however, keep in mind the rapid vasomotor alterations of such animals to outside stimuli and recognize that changes of this magnitude may occur normally. A comparison in anesthetized animals of the response of the blood pressure obtained simultaneously with the tail plethysmograph and a mercury manometer following intravenous injections of sodium nitrite, ephedrine and pitressin showed values that differed by only a few millimeters of mercury.

Summary. The tail plethysmograph as used on the rat has been modified for use on the dog. When properly applied, it affords an easy and repeatable measure of arterial blood pressure in close agreement with values obtained by the mercury manometer.

14914

Nucleic Acid Derivatives as Growth Factors for Certain Group A Hemolytic Streptococci.*

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The development of a satisfactory test for the *in vitro* resistance of Group A (Lancefield) streptococci to sulfonamides has been hampered by the lack of a medium which would support adequate growth of freshly isolated strains and would, at the same time, contain little or no sulfonamide antagonists. It was discovered that the semi-synthetic medium of Roe and Adams[1] fulfilled these desiderata when it was supplemented with 10% normal rabbit serum, and such a medium was made the basis of a method for testing resistance of streptococci to sulfonamides.[2] It was discovered, however, that certain strains which grew well in this medium failed to grow at all when horse serum was substituted for rabbit serum. Other strains grew well whichever serum was used.

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* The opinions expressed in this paper are those of the author and do not necessarily represent those of the Navy Department.

The composition of the Roe and Adams medium has been given elsewhere in detail.[2] It consists of caseine hydrolysate, cystine, tryptophane, dextrose, mineral salts, biotin, pyridoxin, nicotinic acid, calcium pantothenate, thiamine, riboflavine, adenine sulfate, uracil, glutamine, asparagine, choline, thioglycollic acid and buffer salts.

There appeared to be two possible explanations for the failure or horse serum to support growth when it was added to this medium: either it exerted an inhibitory influence on certain Group A strains; or it lacked one or several factors essential to the growth of these strains.

That the former explanation was not the case was shown by the failure of horse serum to have an inhibitory effect when added in concentrations as high as 25% to a mixture of the semi-synthetic medium and the smallest amount of rabbit serum (3%) which would produce maximal growth in the absence of horse serum.

It appeared, therefore, that certain strains of Group A streptococcus required a factor for growth which was lacking in horse serum but which was supplied by rabbit serum. For convenience this was called the RS factor.

The work reported here presents evidence concerning the dependence of various Group A streptococci on the RS factor, the distribution of the factor in certain biological materials and the ability of certain nucleic acid derivatives to replace the factor.

TABLE I.
Dependence of Various Group A Streptococci on the RS Factor According to Serological Type.

Type	No. requiring RS factor	No. not requiring RS factor
1	3	1
2	0	4
3	0	4
4	1	4
5	3	1
6	1	2
10	0	1
11	1	0
13	0	2
14	1	1
17	0	5
18	0	3
19	8	12
23	1	0
24	0	1
26	0	4
28	0	1
29	2	1
30	4	2
All types	25	49

To eliminate the chance that the absence of the factor was a peculiarity of the particular horse which served as a source of serum for these experiments, sera from 5 other horses[†] were tested and all were found to be similar in their lack of the factor.

TABLE II.
Relationship of RS Requirement and Type Antigens of Type 19 Streptococci.

	No. requiring RS factor	No. not requiring RS factor
Strains containing M and T antigens of Type 19	7	8
Strains containing M antigen but no T antigen of Type 19	1	4

Further studies showed that human, pig, dog and sheep sera resembled rabbit serum in supplying the factor, whereas cow, rat and mouse sera lacked it.

For testing the ability of the various sera to support growth of the exacting strains, tubes containing 2 cc of a medium composed of 90% semi-synthetic medium and 10% of the serum to be tested were given inocula of approximately 10^{-5} cc of strains known to require the factor by the technique of MacLeod and Mirick,[3] and were grown at 37° for 24 hr. Control tubes were set up using rabbit serum in place of the unknown sera. Growth was estimated visually and maximal growth compared favorably to that obtained with a very rich medium such as Todd-Hewitt broth.

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[1] Roe, A. S., and Adams, M. H., abstracted in *J. Bact.*, 1944, **47**, 432.

The present author wishes to express his appreciation for permission to use this medium before ful details of its composition have been published by them.

[2] Wilson, A. T., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 130.

[†] These sera were kindly furnished by Dr. L. Earl Arnow of Sharp and Dohme, Inc., Glenolden, Pa.

Peptone and fresh meat infusion from beef and horse muscle were shown to contain the factor in large amounts.

A number of substances which had previously been identified as growth factors for various microorganisms, but which were absent from the Roe and Adams medium, were added individually to the deficient horse serum-semi-synthetic medium and it was discovered that the addition of xanthine in concentrations as low as 0.6 γ per cc was effective in promoting growth of the exacting strains.

Since adenine and uracil were already present in the semi-synthetic part of the medium, it appeared that the need for xanthine represented a specific, or at least an additional, purine requirement.

A survey of representative strains of various serological types of Group A streptococcus[‡] was undertaken in order to determine the frequency of dependence on the RS factor. This dependence was determined by inoculating 10^{-5} cc of blood broth culture into each of 2 tubes which contained respectively 2 cc of a mixture of 90% semi-synthetic medium with 10% horse serum, and 2 cc of the same mixture with xanthine added to a final concentration of 1 mg per 100 cc. Most of the strains could be sharply divided into two classes: those which grew equally well in both media and those which grew well in the xanthine-containing medium but gave no visible growth in the medium to which no xanthine had been added, even after incubation as long as seven days. A few strains grew very slowly in the xanthine-free medium, but final growth, after several days, was equal to that in the medium containing xanthine.

Approximately one-third of the strains required the RS factor (Table I). The number of strains tested for most of the types was small, but the frequent occurrence of strains of both classes within the same type makes it unlikely that there is any association between this requirement and serological type. Studies of strains of Type 19 which had been analyzed for their M- and T-antigens by Dr. Lancefield, and which were kindly supplied by her, showed no correlation between xanthine requirement and the presence of either of these antigens (Table II). It was further shown that there was no association between resistance of a strain to sulfonamide and its dependence on the factor.

No instance was encountered of an exacting strain changing to a non-exacting strain or vice versa. Occasionally strains which were known to be exacting grew out unexpectedly in the deficient medium, but repetition of the experi-

TABLE III.
Ability of Certain Materials to Replace the RS Factor.

Materials having RS factor effect	Materials not having RS factor effect
Xanthine[1]	Adenine[1]
Guanine[1]	Uracil[1]
Hypoxanthine[2]	Cytosine[2]
Guanosine[2]	Thymine[2]
Desoxyguanosine[2]	Uric Acid
Adenosine[2]	Caffeine
Yeast nucleic acid[3]	Alloxan[6]
Fresh beef infusion	Horse serum
Fresh horse infusion	Cow “
Peptone	Rat “
Rabbit serum	Mouse “
Human “	Streptogenin[7]
Pig “	Thymus nucleic acid[4]
Dog “	
Sheep “	
Thymus nucleic acid[4] after degradation by desoxyribonuclease.[5]	

[1] Eastman Kodak Co., Rochester, New York.

[2] From the P. A. Levene Collection of the Rockefeller Institute through the courtesy of Mr. E. B. Smith.

[3] City Chemical Co., New York.

[4] From Dr. A. E. Mirsky of the Rockefeller Institute, New York.

[5] From Dr. M. McCarty of the Rockefeller Institute Hospital, New York.

[6] From Dr. M. Pijoan, Naval Research Institute, Bethesda, Md.

[7] From Dr. D. W. Woolley, of the Rockefeller Institute, New York.

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[3] MacLeod, C. M., and Mirick, G. S., *J. Bact.*, 1942, **44**, 277.

[‡] These strains were generously supplied by Dr. Rebecca C. Lancefield of the Rockefeller Institute Hospital, New York.

TABLE IV.
Effect of Desoxyribonucleic Acid Before and After Treatment with Desoxyribonuclease.

	Strains known to require the RS factor			Strains known not to require the RS factor		Uninoculated
	2848F	2884F	2885F	859GL	861GL	
Desoxyribonucleic acid alone	—	—	—	++++	++++	—
Desoxyribonucleic acid with desoxyribonuclease	++++	++++	++++	++++	++++	—
Desoxyribonuclease alone	—	—	—	++++	++++	—
Inactivated desoxyribonuclease alone	—	—	—	++++	++++	—
Desoxyribonucleic acid with inactivated desoxyribonuclease	—	—	—	++++	++++	—
Neither	—	—	—	++++	++++	—

ment showed that this was due to some unrecognized variation in the experimental circumstances and not to a change in the requirements of the organism. No serious attempt has been made to "train" exacting strains to grow in the absence of the RS factor.

Various other nucleic acid derivatives were tested for their ability to replace xanthine as the growth factor. This was done by adding solutions containing 25 or 100 mg of the test substances to the deficient medium in such quantity that their final concentration in the medium was 1 mg per 100 cc. Controls using the deficient medium alone and the deficient medium with 1 mg of xanthine per 100 cc were used. The tubes were inoculated with 10^{-5} cc of culture of several strains known to require the RS factor, and, as an additional control, similar tubes were inoculated with strains known not to require the RS factor.

It was found that guanine, hypoxanthine, desoxyguanosine, adenosine and a commercial preparation of yeast nucleic acid were active. On the other hand cytosine, thymine, uric acid, caffeine, alloxan and thymus nucleic acid were inactive (Table III). Adenine and uracil were known to be inactive, since they were already present in the semi-synthetic part of the medium.

Quantitative studies of the relative effectiveness of the various derivatives showed, rather surprisingly, that those which had any activity at all were approximately equal in effect, weight for weight, regardless of molecule size. Too much emphasis should not be placed on these quantitative studies since the medium is not completely defined. Small quantities of unknown active substances or of antagonists may be contributed by the serum, and these unknown materials may not bear a uniform relationship to the various derivatives studied.

It is likely that the ribonucleic acid preparation which was used contained small quantities of free nucleotides and nucleosides and its activity may have been due to the presence of these substances. Possible explanations of the contrasting ineffectiveness of desoxyribonucleic acid were that it was uncontaminated with nucleotides and nucleosides, that desoxyribose could not replace ribose in an active molecule or that the more highly polymerized state of the thymus preparation rendered it unavailable as the factor. The effectiveness of desoxyguanosine indicated that the nature of the sugar did not determine activity. When thymus nucleic acid was exposed to the action of desoxyribonuclease it was converted to an active compound (Table IV). It is believed that the effect of this enzyme leads to a degradation no greater than depolymerization.[4] The enzyme preparation itself was inactive as the RS factor, and was unable to convert thymus nucleic acid to an active compound after exposure to a temperature of 60° for 30 minutes.

A preparation of streptogenin, known to be a growth factor for certain streptococci and supplied by Dr. D. W. Woolley, was inactive.

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[4] McCarty, M., personal communication.

Discussion. The effects of nucleic acid derivatives on the growth of various microorganisms have been studied extensively in recent years.[5-17] In the majority of instances the experimental conditions have been such that all growth requirements of the strains could be met by materials of known chemical composition, and the effects of varying the individual constituents could be studied with great precision. Unfortunately in the case of group A streptococci no fully defined medium exists which will grow all strains luxuriantly. Pappenheimer and Hottle[7] made some interesting observations on one Group A strain which they were able to grow in a semi-synthetic medium. This medium differed from the medium of Roe and Adams chiefly in its use of gelatin hydrolysate in place of caseine hydrolysate, in its lack of choline and asparagine, and in its content of added tyrosine and glutathione. The strain they used, C203S, required adenine for growth at atmospheric CO_2 tension but not at increased CO_2 tension. Uracil accelerated the rate of growth but was not essential. They found that adenine could be replaced by guanine, guanosine, guanylic acid, xanthine or hypoxanthine. This strain was tested by the technique presented in this paper and was found not to be an exacting strain with respect to the RS factor.

Although it is likely that xanthine and its equivalents act as growth factors, the fact that these observations have been made in a complex serum-containing medium makes it difficult to be certain of this point. It has been shown[13] that the various purines and pyrimidines may be mutually antagonistic in relation to growth stimulation, and this antagonism has been compared to that existing between para-amino-benzoic acid and sulfonamides, between pantoyl taurine and pantothenic acid, and between pyridine 3 sulfonic acid and nicotinic acid. It is conceivable that xanthine and its equivalents serve merely to counteract the action of some unidentified antagonistic substance which is present in horse serum but not in rabbit serum. Elucidation of this point and particularly of the quantitative relationships of the various derivatives must await the development of a chemically defined medium which will grow Group A streptococci.

In view of the observations of Loring and Pierce[16] that nucleosides and nucleotides were many times more active than their constituent pyrimidines for growth stimulation of *Neurospora* mutants, it is interesting to note that adenosine was effective as the RS factor whereas adenine itself was not. It is hoped that this observation can be repeated with other preparations of adenosine and with adenylic acid.

The ability to divide Group A streptococci of a single serological type into two classes on the basis of their dependence on the RS factor may place another tool in the hands of epidemiologists who find it desirable to "tag" organisms.

Conclusions. 1. Approximately one third of a group of representative Group A streptococci required a factor for growth in a semi-synthetic medium reinforced with serum which was present in rabbit, human, pig, dog and sheep sera. This factor was absent from horse, cow, rat and mouse sera. 2. The factor could be replaced by yeast nucleic acid and certain of its derivatives, such as xanthine, guanine and hypoxanthine. It could not be replaced by certain other derivatives such as

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[5] Richardson, G. M., *Biochem, J.*, 1936, **30**, 2184.

[6] Moller, E. F., *Z. Physiol. Chem.*, 1939, **260**, 246.

[7] Pappenheimer, A. M., Jr., and Hottle, G. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 645.

[8] Snell, E. E., and Mitchell, H. K., *Proc. Nat. Acad. Sci.*, 1941, **27**, 1.

[9] Stokstad, E. L. R., *J. Biol. Chem.*, 1941, **139**, 475.

[10] Mueller, J. H., and Miller, P. A., *J. Biol. Chem.*, 1941, **140**, 933.

[11] Robbins, W. J., and Kavanagh, F., *Proc. Nat. Acad. Sci.*, 1942, **28**, 4.

[12] Robbins, W. J., and Kavanagh, F., *Proc. Nat. Acad. Sci.*, 1942, **28**, 65.

[13] Pennington, D. E., *Proc. Nat. Acad. Sci.*, 1942, **28**, 272.

[14] Feeney, R. E., and Strong, F. M., *J. Am. Chem. Soc.*, 1942, **64**, 881.

[15] Hopkins, F. G., and Simon-Reuss, I., *Proc. Royal Soc.*, Ser. B, 1944, **132**, 253.

[16] Loring, H. S., and Pierce, J. G., *J. Biol. Chem.*, 1944, **153**, 61.

[17] Gibert, E. B., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 363.

adenine and uracil. 3. Desoxyribonucleic acid was inactive as the factor, but became active after depolymerization by desoxyribonuclease. 4. The lack of uniformity of Group A streptococci with respect to dependence on this factor shows that nutritional studies performed on a single strain cannot be regarded as representative of the entire group.

Acknowledgement of indebtedness to the many individuals who have supplied materials for this investigation will be found in the text, in the footnotes and in the tables of this paper. A word is appropriate here concerning the generosity of those who have these rare materials in their possession and who lend them so freely. An undertaking of this sort would be impossible without it. The author wishes further to thank Dr. A. E. Mirsky for his interest and for his many suggestions.

14915 P

Diabetes in a Totally Depancreatized Man.*

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We wish to report observations on the diabetes of a totally depancreatized man. The surgical aspects of the case have been described elsewhere.[1] The patient in question had had spontaneous diabetes, known for 9 months before he entered the hospital, and his father also had had the disease. His presenting complaints were abdominal pain and diarrhea of 2 years' duration and, recently, thirst, polyuria and loss of weight. The symptoms, except for the diarrhea, were largely ameliorated by diet and insulin. While under treatment he developed jaundice and at operation was found to have a carcinoma of the pancreas with infiltration of the neighboring organs. The entire pancreas, duodenum, stomach, spleen, left adrenal and most of the omentum were removed. The liver appeared normal. Histologic examination of the uninvolved head and neck of the pancreas (Dr. George Gomori) showed insular changes characteristic of diabetes. At the conclusion of the operation there was no gross evidence of remaining neoplasm. The postoperative course was uneventful and the patient was out of bed on the sixteenth day. For 6 weeks he was maintained on both protamine zinc and crystalline insulin, but thereafter, in order to facilitate studies, was given crystalline insulin alone. He also received vitamin preparations parenterally, atropine to control diarrhea, and at intervals large doses of powdered pancreas. He survived for 14 weeks after operation, death being due to recurrence of the carcinoma and, terminally, to the withdrawal of insulin.

Insulin Requirement. Before operation, on a diet of C 398, P 200, F 12 g (glucose equivalent 511 g), the amount of insulin necessary to keep the urine nearly sugar-free was from 40 to 65 units per day. The body weight at this time was 72 kg (158lb). Two months after operation, when the patient was up and about, the diet was C 401, P 102, F 11 g (glucose equivalent 459 g) and the insulin requirement was about 40 units per day. The body weight was now 56 kg (123 lb). The apparent reduction in the demand for insulin may have been due to 2 factors: first, the lower glucose value of the postoperative diet, and second, the loss of 16 kg of weight. The striking fact, however, is not that the insulin requirement was lower, but that it was no higher.

Sensitivity to insulin was marked, the patient showing frequent hypoglycemic reactions.

Withdrawal of Insulin. Approximately 3 months after operation all insulin was withdrawn for a period of 4 days. On the first day, glycosuria, previously less than 10 g per day, rose to 112 g. On the second day, the value was 143 g and ketosis appeared. On the fourth day, all food was withheld and the urine showed a D/N ratio of 2.42. Insulin was reinstituted that evening because of severe thirst and discomfort. The patient had lost 4.5 kg of weight during this period.

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* This study was aided by a grant from the Charles H. and Mary F. S. Worcester Memorial Fund of the University of Chicago.

[1] Brunschwig, Alexander, and Ricketts, Henry T., *Surg. Gyn. and Ob.*, 1945, in press.

Six days later, with the patient's appetite failing, insulin was again withdrawn. Marked glycosuria and ketosis promptly appeared. Two days later, the urine contained 108 g of glucose and the patient stopped eating entirely. On the fourth day of this period, the blood sugar was 570 mg %, and between the fifth and sixth days the urine showed a D/N ratio of 3.59. Death occurred on the sixth day in typical diabetic coma.

Fasting respiratory quotients were determined daily for 5 days when the patient was in relatively good health and was receiving crystalline insulin 3 times per day, the last dose being given before supper. The values varied from 0.77 to 0.80. Fasting blood sugars on the same mornings were in the neighborhood of 100 mg per 100 cc. Determinations of the R.Q. during the complete withdrawal of insulin were unsatisfactory.

Fat Metabolism. The serum lipids, which before operation ranged from 800 to 1260 mg per 100 cc, slowly declined after pancreatectomy but never sank to subnormal levels, the minimum being 707 mg per 100 cc. At autopsy the liver was grossly fatty, probably owing to the patient's death in diabetic coma.

Conclusions. (1) The pancreas, or its hormone, insulin, is necessary to life in the human. (2) On the basis of the present case and others previously reported,[2-5] the insulin output of the normal human pancreas must be considerably smaller than has been believed and seems to lie in the range of 30 to 50 units per day. Therefore, (3) in diabetic patients requiring more than this amount, an extra-insular factor must be contributing to the diabetic syndrome. (4) The D/N ratio of from 2.42 to 3.59 indicates that a fasting, depancreatized man deprived of insulin converts protein to carbohydrate at a rate commensurate with that observed in the dog under the same conditions[6] or when treated with phloridzin.[7]

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[2] Rockey, Eugene W., *Ann. Surg.*, 1943, **118**, 603.

[3] Goldner, Martin G., and Clark, Dwight E., *J. Clin. Endocrinol.*, 1944, **4**, 194.

[4] Priestley, James T., Comfort, Mandred W., and Radcliffe, James, Jr., *Ann. Surg.*, 1944, **119**, 211.

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14916

Effect of p-Aminobenzoic Acid on the Growth of Typhus Rickettsiæ in the Yolk Sac of the Infected Chick Embryo.*

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The search for chemical compounds having possible therapeutic effects on typhus fever, which centered first on sulfonamide derivatives, has been extended to include all kinds of substances having effects on pathogenic bacteria and protozoa. Snyder, Maier, and Anderson[1] tested the activity of a large number of drugs (sulfonamides, anti-luetic, anti-malarial, and trypanosomicidal compounds) on the

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* Report to the Director of the United States of America Typhus Commission, December 16, 1943, withheld from publication. Since this time two other papers dealing with the therapeutic effect of P.A.B.A. were published.

† Member of the United States of America Typhus Commission.

TABLE I.
Effect of Paba on Survival Time of Typhus-Infected Embryos.

		Total No. of embryos	No. of daily deaths Day: 3	4	5	6	7	8	9	10	11	12	Survived	% dead
Epidemic Typhus	Controls	43	3	10	14	13	3						0	100
	Paba (4 mg)	44	0	10	6	4	2	5	2	1	3	2	9	79.5
Murine Typhus	Controls	25	10	2	3	8	2						0	100
	Paba (4 mg)	23	3	5	4	2	2	1					6	73.9

TABLE II.
Effect of Paba on Number of Rickettsiæ.

		Total No. of Eggs	No. of Rickettsiæ per field: 0 No.	0 %	1-10 No.	1-10 %	100 No.	100 %	1000 No.	1000 %	Countless No.	Countless %
Epidemic	Controls	56	7	12.5	29	51.8	8	14.3	8	14.3	4	7.1
	Paba	59	42	71.2	16	27.1	1	1.7	0	0	0	0
Murine	Controls	30	0	0	12	40	5	16.6	5	16.6	8	26.7
	Paba	27	14	51.9	9	33.3	2	7.4	1	3.7	1	3.7

survival time of mice and cotton rats which had been infected intraperitoneally with typhus. None of these substances had beneficial effects, but their experiments led to the trial of p-aminobenzoic acid, which gave indications that it might suppress the growth of typhus rickettsiae. More recently, Andrewes, King, and Van den Ende[2] reported that, of more than 164 compounds which were being tested, only 3 showed anti-rickettsial activity as measured by the ability of the drugs to prevent or retard the development of pulmonary lesions in intranasally-inoculated mice. A preliminary experiment by these authors[2] with p-aminobenzoic acid tended to confirm the findings of Snyder, Maier, and Anderson, in that maximal tolerable doses of the drug caused a reduction in the number and size of pulmonary lesions.

In view of the availability of p-aminobenzoic acid (hereafter referred to as paba) and its suggested therapeutic action on typhus in mice, we decided to test its effect directly on the growth of rickettsiae by inoculating it into the yolk sac of the developing chick embryo.

Procedure. Eggs were selected which contained normally-developing embryos after 6 days' incubation. These were divided into two equal lots. The first or control group, was inoculated into the yolk sac with 1 cc of physiological salt solution per egg, and the other group received an equal volume of salt solution containing paba. The drug was used initially in two concentrations: 4 mg/cc (in solution), and approximately 25 mg/cc (suspended crystals). On the following day each embryo, in both the control and treated series, was inoculated in the yolk sac with 0.5 cc of infective typhus material. The inoculum was prepared from either epidemic (Breinl) or murine (Wilmington strain) infected yolk sacs. These were ground with alundum and diluted 1:20 with physiological salt solution.

The data were recorded in either of two ways, according to the type of analysis which was to be made: (1) The survival time of treated eggs as compared with controls was measured by recording all deaths, beginning on the third day after inoculation with rickettsiae and extending up to hatching. Smears made from the yolk sacs of dead embryos were stained by Machiavello's method and examined for rickettsiae. Or, (2) all eggs in both treated and control series were opened after an arbitrary length of time (3-6 days) and examined for rickettsiae. Counts were made according to the following system: O, + =

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[1] Snyder, J. C., Maier, J., and Anderson, C. R., Report to The Division of Medical Sciences, National Research Council, Washington, D.C., Dec. 26, 1942.

[2] Andrewes, C. H., King, H., and Van den Ende, M., *Brit. Rep. from Nat. Inst. for Med. Res.*, Hampstead, London, 1943.

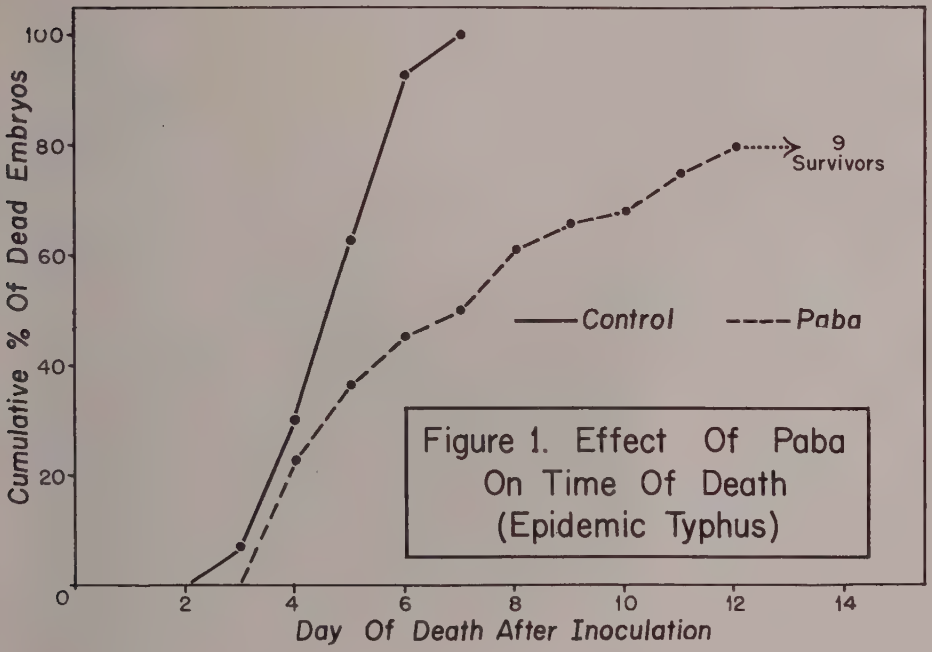


Figure 1. Effect Of Paba On Time Of Death (Epidemic Typhus)

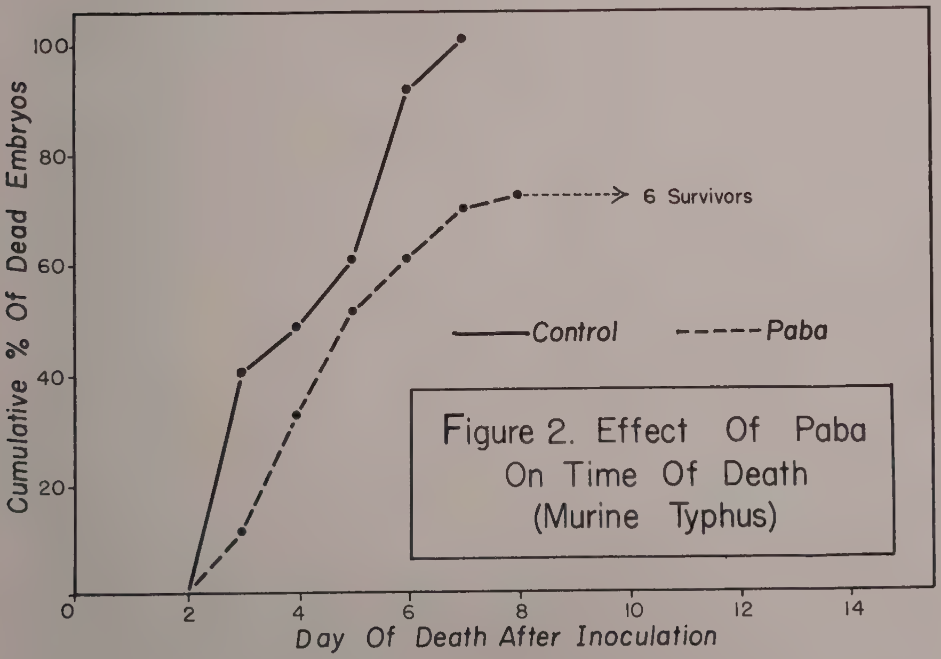
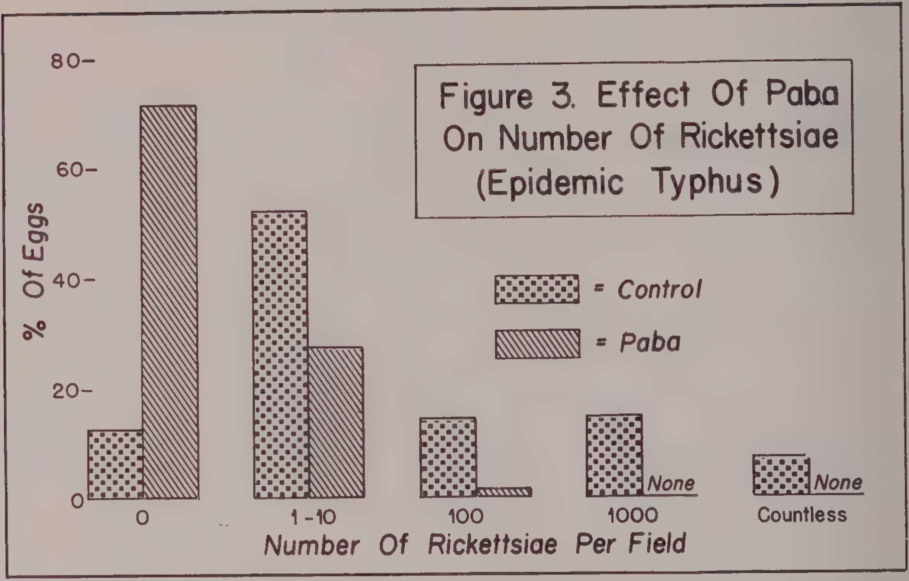
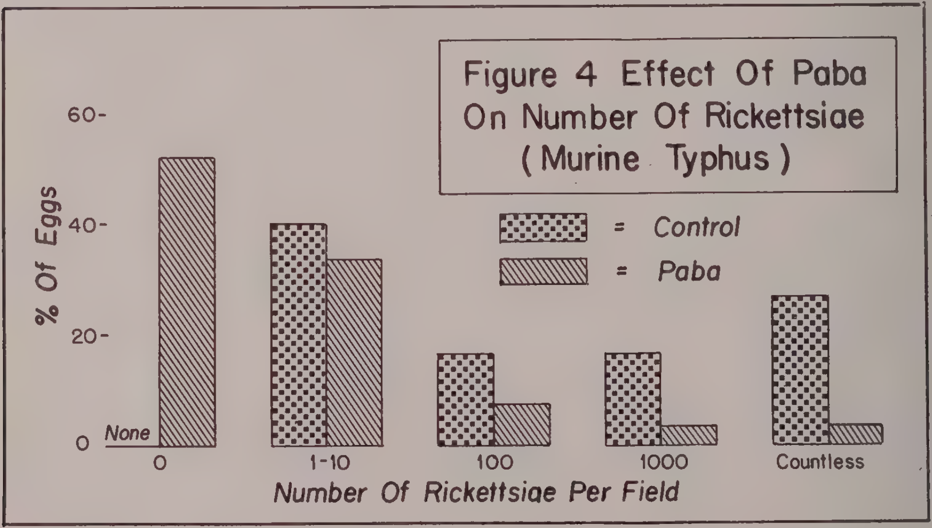


Figure 2. Effect Of Paba On Time Of Death (Murine Typhus)





1-10, ++ = 100, +++ = 1000, ++++ = countless rickettsiae per field.

This method of testing for chemotherapeutic effects on rickettsiae in the yolk sac of the developing chick embryo has several advantages: (1) it permits rather precise measurements of the action of chemicals on rickettsial growth by making rickettsial counts; (2) the action of the drugs can be tested with equal ease on either murine or epidemic strains of typhus; (3) dosages can be carefully controlled.

Prolongation of Survival Time by Paba. When the inoculated embryos are allowed to develop until death occurs from rickettsial infection, a rather striking difference becomes apparent between the control and paba-treated series. Table I shows the distribution of deaths in both series for a total of 87 eggs

Figure 5 Effect Of Delayed Treatment With Paba On Survival Time (Epidemic Typhus)

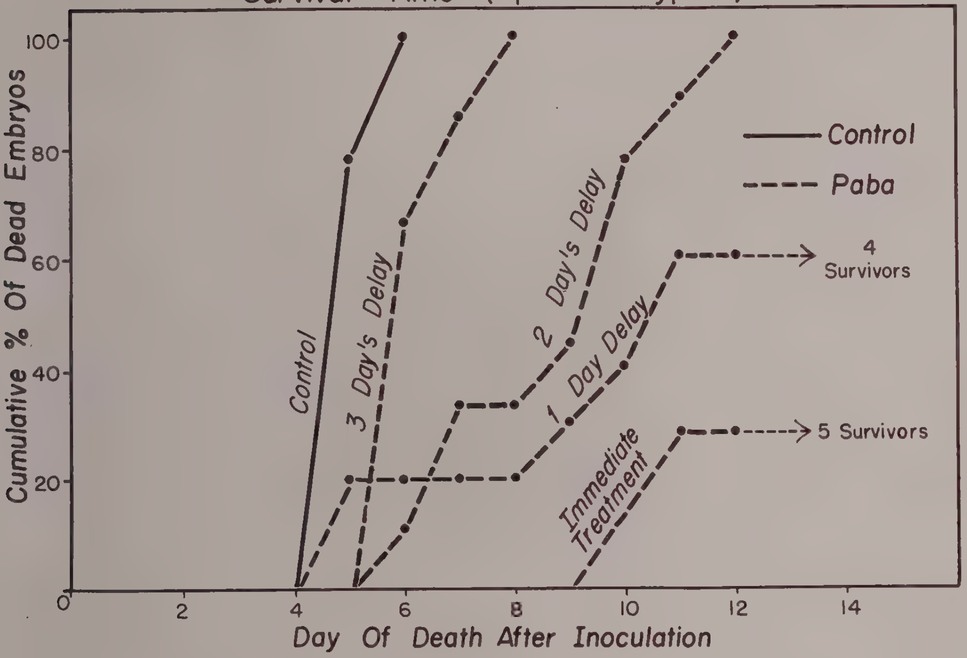


TABLE III.
Effect of Different Concentrations of Paba on Survival of Chick Embryos Infected with Epidemic Typhus.

	Concentration of paba	Total No. of embryos	Days: 3	4	5	6	7	8	9	10	11	12	13	Avg. day of death
			No. of daily deaths											
Exp. No. 1	0 mg	10	0	1	3	4	2							5.7
	0.04	11	0	0	5	5	1							5.6
	0.2	11	0	0	0	11								6.0
	2.0	6	0	0	0	0	0	0	2	0	0	0	4	killed to prevent hatching
Exp. No. 2	0 mg	10	5	4	1									3.6
	0.2	12	0	12										4.0
	0.4	12	2	5	3	1	1							4.5
	2.0	9	1	1	3	1	1	0	0	0	0	0	2	killed to prevent hatching
Exp. No. 3	0 mg	11	1	2	8									4.6
	0.4	14	0	3	2	5	3	0	1					5.85

which were infected with epidemic typhus. It will be noted that all deaths in the control series occurred between the third and seventh days following inoculation with rickettsiae. In contrast to this "bunching" of deaths in the controls, the paba-treated embryos showed only a moderate mortality on the fourth and fifth days, followed by sporadic deaths up to the thirteenth day (twentieth day of incubation). At this time there were still 9 survivors, which were killed to prevent hatching. When the cumulative % of daily deaths is plotted against time, the lag in time of death produced by paba may be shown graphically (Fig. 1).

Similar results were obtained when the eggs were infected with murine rickettsiae (Table I;

TABLE IV.
Effect of Isomers of Paba and Other Substances on Time of Death of Typhus-Infected Embryos.

Compound	Total No. embryos	Days: 3	4	5	6	7	8	9	10	11	12	% dead	Avg. day of death
				No. of daily deaths									
o-Aminobenzoic acid	11	0	4	2								100	4.2
Controls	6	1	3	2								100	4.2
m-Aminobenzoic acid	9	3	0	6								100	4.3
Controls	9	6	0	3								100	3.7
Acetyl p-aminobenzoic acid (Seitz filtered)	13	0	7	4	2							100	4.6
Controls	11	0	4	7								100	4.6
Acetyl p-aminobenzoic acid (heat sterilized)	9	0	0	0	0	2	1	0	3	1	2*	77.8	....
Controls	8	0	0	4	3	0	1					100	5.8
Sulfanilamide	10	3	0	6	1							100	4.5
Controls	10	1	2	6	1							100	4.7
Sodium benzoate	10	1	5	4								100	4.3
Controls	13	2	3	7	1							100	4.5
Benzoic acid	12	7	5									100	3.4
Controls	7	4	1	2								100	3.7

* Survivors.

TABLE V.
Effect of Delayed Treatment with Paba on Survival of Typhus-Infected Embryos.

	Total No. embryos	Day: 1-4	5	6	7	8	9	10	11	12	Survived	% dead
				No. of daily deaths								
Immediate treatment	7	0	0	0	0	0	0	1	1	0	5	28.6
1 day delay	10	0	2	0	0	0	1	1	2	0	4	60
2 " "	9	0	0	1	3	0	1	2	1	1	0	100
3 " "	7	0	0	5	1	1					0	100
Controls	9	0	7	2							0	100

Fig. 2). Here again, all control deaths occurred between the third and seventh days. However, death was considerably delayed in the paba-treated group, with 6 embryos surviving to hatching.

An examination of smears made from these dead paba-treated eggs showed that there were usually very few rickettsiae present. However, some of the eggs which died at a later date did contain increased numbers of rickettsiae. Apparently, therefore, the drug acts by suppressing the growth rate of the rickettsiae, so that appreciable numbers develop only after a long incubation period. Further evidence for this interpretation was obtained by comparing the numbers of rickettsiae in treated and control eggs.

Reduction in Number of Rickettsiae. Counts were made of the number of rickettsiae per oil immersion field in yolk sac smears from 86 controls and 86 paba-treated eggs, and tabulated in 5 categories as shown in Table II. When epidemic rickettsiae were inoculated into control eggs, over 50% contained at least 1-10 rickettsiae per field when examined 3-6 days after infection. Lesser percentages contained 100, 1000, and countless rickettsiae, as shown in Fig. 3. In contrast to this distribution of controls, over 70% of the infected paba-treated eggs contained no rickettsiae when examined after the same period of incubation. Twenty-seven % showed 1-10 rickettsiae, and only 1 egg had as many as 100 per field (Fig. 3).

An exactly comparable picture was obtained when the eggs were infected with murine rickettsiae. In general, the controls were richer than when the epidemic strain was used, but the paba-treated eggs were predominantly negative or in the 1-10 group (Fig. 4). Only 1 egg was classified in each of the 2 highest categories (1000, countless).

It is clear from these data that an inhibition of rickettsial growth is produced by the use of paba. This inhibition probably accounts in part for the delay in the death of the infected embryo.

Specificity of the Paba-Inhibition. The data thus far reported were obtained by using relatively enormous amounts of paba (4 mg per egg, = approximately 70γ/cc, or more) compared with the concentrations which are sufficient to affect bacterial growth (fractions of a gamma per cc[3,4]). In fact, 4 mg was the maximal dose that 6-day embryos could tolerate; larger amounts caused death in 5-8 days. When the concentration of paba was reduced to 2 mg per egg, or less, results were obtained as shown in Table III.

It is seen that the least amount of paba which will cause delay in death of the embryo is 0.4 mg per egg. The inhibition of rickettsial growth is slight, however, for a comparison of smears from the treated and control eggs shows no conclusive difference in numbers of rickettsiae. With more massive dosages, the inhibition of rickettsial growth becomes more or less complete.

The necessity for using such large quantities of paba raises the possibility that a non-specific inhibition might be involved. It is well-known that massive amounts of a chemical (particularly a growth stimulant) may produce unfavorable conditions for growth, regardless of its effect (or lack of activity) in low concentrations. Accordingly, the two isomers, ortho- and meta-aminobenzoic acid, as well as acetyl p-aminobenzoic acid (both heat-sterilized, and sterilized by Seitz-filtration), sulfanilamide, sodium benzoate, and benzoic acid were tested in concentrations equivalent to the maximal dose of paba (4 mg per egg). None of these substances had any apparent effect either on time of death (Table IV) or number of rickettsiae, with the exception of the heat-sterilized acetyl derivative. This was of particular interest, because heating of acetyl paba is known to convert a certain proportion of the compound into paba, by hydrolysis. It seems clear, therefore, that the inhibition of growth of typhus rickettsiae by paba is due to a specific action of the drug.

Effect of Delayed Treatment with Paba. In connection with its possible use as a therapeutic agent, it seemed of value to know what degree of inhibition would be produced by paba if treatment were delayed beyond the time of infection. Five groups of eggs were injected with sufficient rickettsiae (Breinl strain) to cause death in 4-6 days. One group was retained as a control series, and received an additional injection of physiological salt solution immediately after infection. Another group received a similar volume (0.5 cc) of salt solution containing 2 mg of paba. The remaining 3 groups were injected with 2 mg of paba 24 hr, 48 hr, and 72 hr after infection, respectively. The results are shown in Table V and Fig. 5.

It will be noted that the efficacy of paba becomes progressively less, the longer the delay between infection and treatment, as might be expected. However, there is a surprising amount of protection when treatment is begun as late as 48 hr after infection. A similar gradient was obtained with regard to reduction in the number of rickettsiae. When treatment was delayed for 72 hr there was little, if any, reduction in number, but with 48 and 24 hr delays the smears were reduced from ++++ to +, and there was almost complete inhibition with immediate treatment. The data indicate, therefore, that good results may be obtained as long as 48 hr after infection, although earlier treatment is required for best protection.

Discussion. It is to be noted that the percentage of embryos protected by 2 mg of paba was, in general, higher than when 4 mg was used. For example, with 2 mg of paba, there were 22%, 66%, and 71% survivors (Tables III and V) as compared with 20% and 26% survivors with 4 mg (Table I). Since no rickettsiae were observed in a considerable number of the eggs which died in spite of the 4 mg, it seemed possible that some of these deaths might be attributed to a delayed toxic action on the part of the paba rather than to failure of the paba to inhibit rickettsial growth. While not completely studied, evidence for this interpretation was found in a later experiment with non-infected embryos which had been treated with 4 mg of paba. Although normal

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[3] Landy, M., and Dicken, D. M., *J. Biol. Chem.*, 1942, **146**, 109.

[4] Rubbo, S. D., and Gillespie, J. M., *Lancet*, 1942, **242**, 36.

development of the embryos took place during the first week, the drug apparently caused some permanent damage in that a significant number of them became moribund and died during the latter part of the incubation period. Because of this delayed toxicity of the maximal dosage, it is probable that some of the embryo deaths shown in Fig. 1 and 2 are attributable to the drug and not to the rickettsiae.

In addition to its activity against typhus rickettsiae, preliminary experiments have shown that paba also inhibits growth of the rickettsiae of Rocky Mountain spotted fever, but has no apparent effect on growth of psittacosis elementary bodies. Further experiments are now in progress on the mode of action of p-aminobenzoic acid.

Conclusions. I. p-Aminobenzoic acid inhibits the growth of typhus rickettsiae and prolongs the survival time of infected chick embryos. The inactivity of related compounds indicates that the inhibition is specific to paba. 2. The minimum amount of paba which will inhibit rickettsial growth is 0.4 mg per egg. In general, the inhibition is more complete with maximal dosages, which in the case of the 6-day chick embryo is 2-4 mg. These data suggest that relatively massive amounts of paba may have to be administered if beneficial effects are to be expected on human cases of typhus. 3. When treatment with paba is delayed 24, 48, and 72 hours beyond the time of infection, there is progressively less delay in the time of death and less reduction in the number of rickettsiae. However, good results are obtained with treatment as late as 48 hr after infection.

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Penicillin as a Sporicidal Agent.

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Although Fleming's[1] first paper on penicillin furnished evidence of bactericidal as well as bacteriostatic activity, until 1941 attention was focused almost exclusively on the bacteriostatic or growth-inhibiting action of the drug.[2-4] Dawson *et al.*[5] reported that "Penicillin appears to be bactericidal against the pneumonococcus *Streptococcus viridans,* staphylococcus and *Clostridium welchii, Cl. sporogenes,* and *Cl. septique*" (the latter three in the vegetative form). More recent reports have revealed strong bactericidal activity by penicillin against *Streptococcus hemolyticus,*[6-9] *S. viridans,*[10] pneumococci,[6,7] staphylococci,[7,11] and meningococci.[12]

Investigators[6,9,12,13] are essentially in agreement that penicillin activity is demonstrable only when the organisms are in an environment conducive to multiplication and it has been shown[14] that the more active the multiplication the greater the effect of penicillin.

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[1] Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 226.

[2] Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., On-Wing, J., and Sanders, A. G., *Lancet*, 1940, **2**, 226.

[3] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

[4] Reid, R. D., *J. Bact.*, 1935, **29**, 215.

[5] Dawson, M. H., Hobby, G. L., Meyer, K., and Chaffee, E., *J. Clin. Invest.*, 1941, **20**, 434.

[6] Hobby, G. L., Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 277.

[7] Dawson, M. H., Hobby, G. L., Meyer, K., and Chaffee, E., *Ann. Int. Med.*, 1943, **19**, 707.

[8] Robinson, H. L., *J. Pharm. and Exp. Therap.*, 1943, **77**, 70.

[9] Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 178.

[10] Watson, R. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 66.

[11] Rantz, L. A., and Kirby, W. M., *J. Immun.*, 1944, **48**, 335.

[12] Miller, C. P., and Foster, A. Z., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 205.

Since non-sporulating or vegetative bacteria seem to respond to penicillin only under narrowly circumscribed conditions of environment and physiologic activity, it is hardly surprising that bacterial spores have received no attention in penicillin studies.* Apparently it has been assumed that penicillin has no sporicidal activity. Observed facts, however, do not support this assumption. In this communication we shall show that spore suspensions may undergo a marked reduction in numbers when they are incubated for some hours with a relatively low concentration of penicillin.

We have previously shown† that in many aerobic species spores remain in the unchanged heat stable condition under favorable conditions for growth unless they have been previously heated for a few minutes at sublethal temperatures. The latter process termed heat activation promotes the change from the heat stable to the heat labile condition which is normally followed by vegetative development.

The action of penicillin upon bacterial spores in distilled water and in milk is shown in Table I. Washed spores (entirely free from vegetative cells except 1518) were added to and thoroughly mixed with the sterile test medium, then heated mildly for a few minutes to precondition them for germination as described in the preceding paragraph. Penicillin‡ (in a 5% glucose solution) was then added and allowed to act at a favorable temperature for several hours. At the end of the reaction period the residual penicillin was completely inactivated by means of a standardized sterile solution of penicillinase.§ The difference between the numbers of viable spores as determined by pour plates in the test and control suspensions before and after incubation is the measure of the sporicidal activity of the penicillin. Table I shows that with one exception penicillin effected a marked reduction in the number of viable spores when the periods of contact were 5 and 27 hours. The two strains of *B. subtilis* were the most susceptible. *B. cereus* apparently was unaffected. In both subtilis strains and *B. megatherium* the destruction of the spores was much greater in milk than water. This is contrary to usual experience wherein germicidal activity is almost universally diminished by the presence of organic matter. The greater activity of penicillin against the spores of *B. subtilis* and *B. megatherium* in milk seems to be accounted for by the data presented in Table II.

In this experiment a sample of milk was uniformly seeded with the spores of 2 strains of *B. subtilis*. Each inoculated sample was divided into 4 equal portions, 2 of these were heated to 95° for 15 min and 2 served as non-heated controls. One each of the heated and non-heated samples were treated with penicillin (5 u/ml), the remaining 2 served as non-penicillin controls. All samples were then incubated at 45° C. At the periods indicated samples were heated at 85° C for 10 min, treated with penicillinase, and plated. The data (Table II) show that the spores became heat labile at the same rate both with and without penicillin; also that the spores remained partially or wholly in the heat stable condition when they had not previously been heated to 95° for 15 min, whether they were exposed to penicillin or not.

The data in the last two columns (Table II) give the total number of viable organisms after the indicated periods of contact with penicillin. These figures compared with the numbers of heat stable spores (next two columns) show that the spores became heat labile much more rapidly than they were killed by penicillin. This lag between conversion of the spores to the heat labile state and evidence of penicillin activity suggests that in sensitive species a nutrient medium promotes penicillin action by permitting the spore to change to a more vulnerable condition at which time development

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[13] Dawson, M. H., Hobby, G. L., Meyer, K., and Chaffee, E., *J. Clin. Invest.*, 1941, **20**, 434.

[14] Hobby, G. L., and Dawson, M. H., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 181.

* Foster, J. W., and Woodruff, H. B. (*J. Biol. Chem.*, 1943, **148**, 723.) recommended the use of *B. subtilis* spores in the cup assay of penicillin, but with no suggestion that sporicidal activity might be involved.

† *J. Bact.*, in press.

‡ Penicillin sodium, kindly supplied by Chas. Pfizer and Co., Brooklyn, N. Y.

§ Obtained through the courtesy of the Schenley Research Institute, Lawrenceburg, Indiana.

TABLE I.
Sporicidal Activity of Penicillin 5u/ml in Water* and in Milk.*

Culture	Medium	Temp. of reaction. °C	No. of viable spores per ml after contact with penicillin 0 hr × 1000	5 hr × 1000	27 hr × 1000
B. subtilis (LB)	Water	45	129	114	15.8
	Milk	45	261	1.6	0.1
" " (6)	Water	45	257	203	42
	Milk	45	256	1.6	0.04
B. megatherium (696)	Water	30	97	81	55
	Milk	30	141	32	8.7
B. cereus (232)	Water	30	67	71	43
	Milk	30	165	1,200[1]	Millions[1]
B. stearothermophilus (1518)	Water	55	173	23	17
	Milk	55	166	24	11

* Suspensions heated at 95° C for 15 min before penicillin added.
[1] Spores and vegetative cells.
u = Oxford units.

TABLE II.
Changes in the Heat Stability of Heated and Non-heated Subtilis Spores When Incubated in Milk With and Without Penicillin. The Total Number of Organisms Surviving Penicillin Action When the Spores Were Previously Heated.

	Number of heat stable spores per ml when treatment was: No penicillin				Penicillin 5u/ml				Total viable organisms per ml Penicillin 5u/ml	
	No heat before incubation—85° for 10 min after		95° for 15 min before incubation—85° for 10 min after		No heat before incubation—85° for 10 min after		95° for 15 min before incubation—85° for 10 min after		95° for 15 min before incubation —no heat after	
Period of incubation min.	LB × 1000	6 × 1000	LB × 1000	6 × 1000	LB × 1000	6 × 1000	LB × 1000	6 × 1000	LB × 1000	6 × 1000
0	39	46	40	46	44	42	44	42	44	42
5			39	34			40	33		
10			31	19			28	20	43	42
15			16	15			17	17	44	43
30			2	6			2	7	44	42
60	43	26	0.2	2	41	26	0.2	2	36	35
120	39	19	0.1	1	42	18	0.2	0.3	9.7	10.2

LB and 6 = strains of *B. subtilis.*
Incubation temperature 45° C.

is apparently arrested and the organisms killed. The development of the spores to the heat labile stage is manifestly not necessary for all spores, since many are killed by penicillin in a non-nutrient medium (Table I). However, with sensitive species it may be easily demonstrated that preliminary mild heating of the spores in water increases their susceptibility to penicillin. Although the heat stability of these heated spores is unchanged, it is apparent that they have undergone certain germinative changes and represent therefore a stage of development which is intermediate between the untreated and heat labile stage. The relationship between susceptibility to penicillin and preliminary heating of spores is brought out in Table III. The effect of heat is pronounced with the Subtilis strains and with *B. megatherium.* The lower count after initial heating of the spores both of *B. megatherium* and *B. cereus* is evidence of killing by heat. With 1518, however, the initially lower count after heating is caused by the elimination of vegetative cells, which are difficult to exclude from the spore cultures of this organism. The check plates with 1518 in the presence of penicillin were inexplicably erratic.

Many, perhaps all, of these cultures produce penicillinase; hence to be effective, penicillin must prevent any considerable vegetative growth after germination of the spores. In suspensions of *B. cereus* spores, penicillin is

TABLE III.
Sporicidal Activity of Penicillin for Heated and Non-heated Spores.

	Penicillin 5u/ml added to milk—viable spores per ml					
	Non-heated spores			Heated spores[1]		
Culture	0 hr × 1000	5 hr × 1000	27 hr × 1000	0 hr × 1000	5 hr × 1000	27 hr × 1000
B. subtilis (LB)	14.7	29.7	4.2	293	2.4	0.03
	(293)[2]	(148)	(61)	(290)	(0.5)	(0.04)
" " (6)	205	28.5	2.1	241	0.8	0.06
	(241)	(19)	(2.7)	(241)	(0.4)	(0.1)
B. megatherium (696)	231	86	23	116	25	6.2
B. cereus (232)	267	2,500[3]	Millions	164	1,200[3]	Millions
B. stearothermophilus (1518)	292	96	18.5	196	108	16.6

[1] 95°-15 min for LB, 6 and 1518; 85°-15 min for *B. megatherium* and *B. cereus.*

[2] Figures in parentheses show plate count after 95°-15 min.

[3] Both spores and vegetative cells.

Incubation temperature: *B. subtilis,* 45° C; *B. megatherium* and *B. cereus,* 30° C; *B. stearothermophilus,* 55° C.

clearly unable to prevent vegetation of germinated spores. Whether this is due to pencillinase production in the intermediate stages of germination or to other factors is not known. Increasing the concentration of penicillin or incubation of the suspensions containing it for longer periods, only slightly increased its sporicidal activity, indicating that even in susceptible species there exists a very small fraction of spores which are relatively resistant to penicillin.

The extraordinary effectiveness of penicillin in low concentration against certain types of spores, together with its non-toxic nature, suggests potential usefulness in non-medical fields, including that of food preservation. The possibility of employing penicillin in conjunction with mild heating merits consideration. Within a very limited sphere of observation, we have observed that a high degree of resistance to penicillin is correlated with a low degree of thermal resistance. Our observations upon the sporicidal activity of penicillin are being extended and will be reported later.

Summary. Under suitable conditions of incubation, penicillin in relatively low concentration is actively sporicidal against many aerobic species of bacteria. In sensitive species the sporicidal activity of penicillin is much greater in milk than in water and is usually enhanced by sublethal heating of the spores before treatment with penicillin.

14918

Electron Shadow-Micrography of Virus Particles.*

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The wave length of the light used with an optical microscope fixes the size of the smallest detail that it will resolve. For the shortest ultraviolet light it is practical to employ, this is in the neighborhood of 0.15 micron. The electron microscope, however, has such a great ultimate resolving power that, at least for the present, the smallest object it will reveal has a size determined by other factors. Photomicrographs produced with electrons are essentially shadow-graphs in which regions that are relatively light on the original negative correspond to regions in the sample which deflect (scatter) a larger percentage of the incident electrons,

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* Aided in part by a grant from the National Foundation for Infantile Paralysis, Inc.

and as long as the problem is not complicated by the crystallinity of the object this ability to scatter depends on the amount of matter in the way of the electron beam. It is found in practice that particles of biological origin often cannot be observed simply because they do not contain enough matter to deflect a detectable percentage of the incident electrons. The problem of extending and improving electron microscopic vision of such small objects can therefore be said to be one of increasing the contrast between different parts of a preparation and between the preparation and the substrate that supports it. One way to do this is by staining with some heavy metal or radical which is selectively adsorbed. Staining with phosphotungstic acid has already been effectively used in the electron microscopy of tendon and muscle.[1] We have approached the problem from a different point of view and have found that "shadow-casting" with a thin film of metal deposited *in vacuo* is a very effective way of revealing the shape as well as the presence of many minute biological objects. The present note illustrates the application of this method to the examination of suspensions of purified viruses, as represented by influenza and the tobacco mosaic virus protein.

Shadow-casting was originally developed[2] as a way of measuring the heights of objects under the electron microscope. It consists in depositing an exceedingly thin (ca 7 mμ) layer of metal obliquely on to the preparation to be examined in the microscope. High points on the surface intercept more than an average number of the condensing atoms and at the same time shield areas immediately behind them. These shielded areas, devoid of deposited metal, appear as "shadows" of the elevated regions responsible for them. Just as an observer on a mountain-top or in a plane sees many details of a landscape at sun-rise,

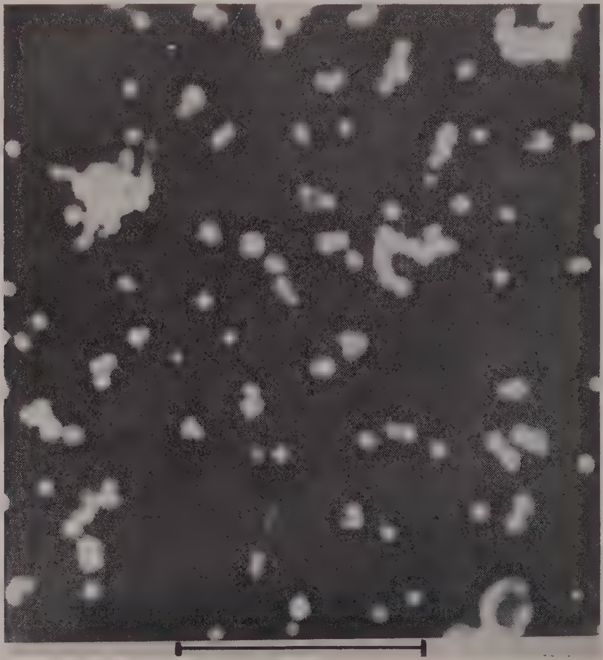

FIG. 1.
An electron micrograph of a suspension of purified PR-8 influenza virus. The numerous round bodies, and their aggregates, are what are now generally considered to be the elementary virus units. In this and each of the other photographs the inscribed line has a length on the photograph corresponding to one micron on the sample.

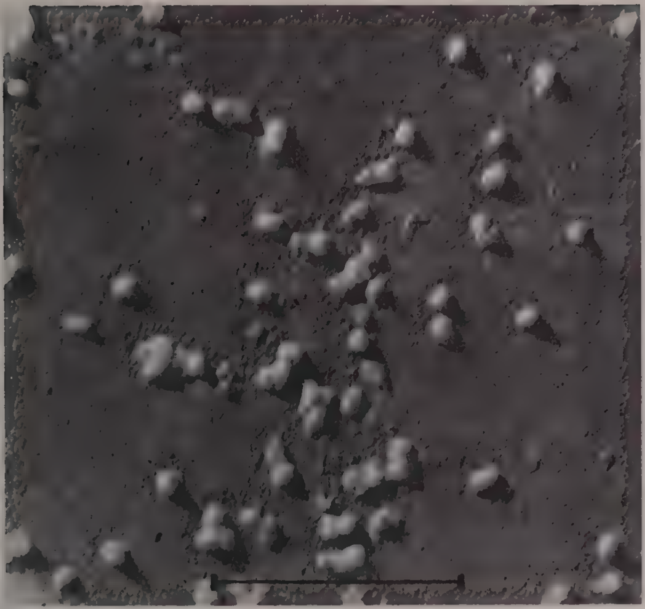

FIG. 2.
A micrograph of a similar preparation after it has been shadowed by the oblique deposition upon it of a thin layer of chromium.

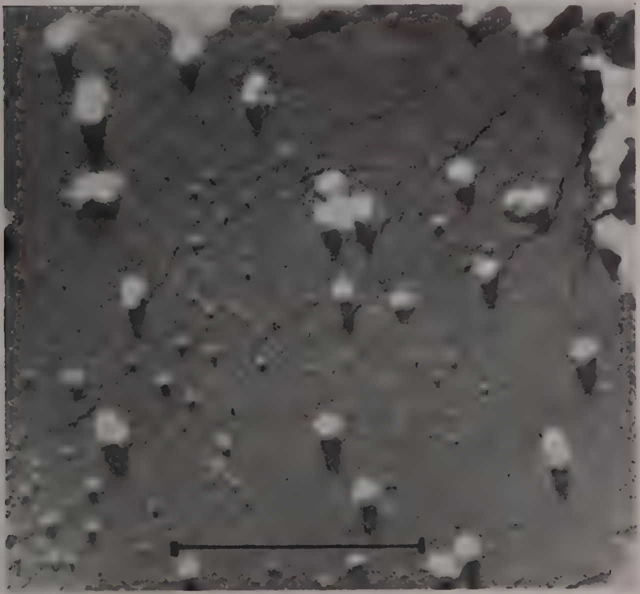

FIG. 3.
A field in another similarly shadowed influenza preparation.

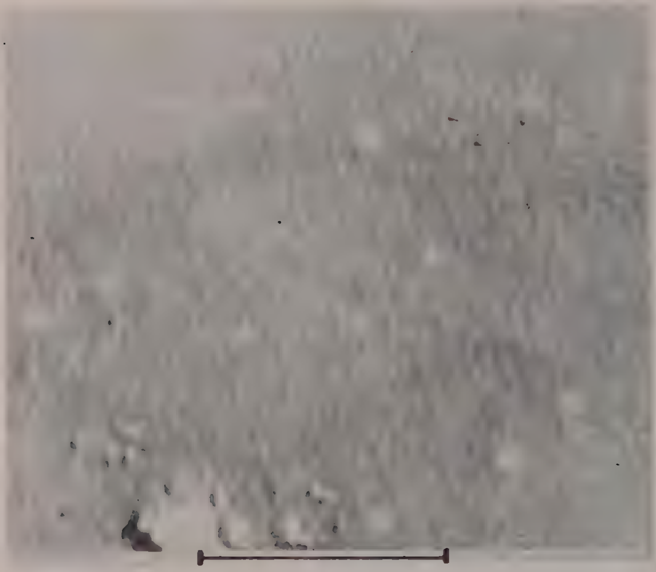

FIG. 4.

A micrograph of a shadowed blank screen to show the texture of the substrate. This field was chosen to include some dirt which appears at the bottom of the picture.

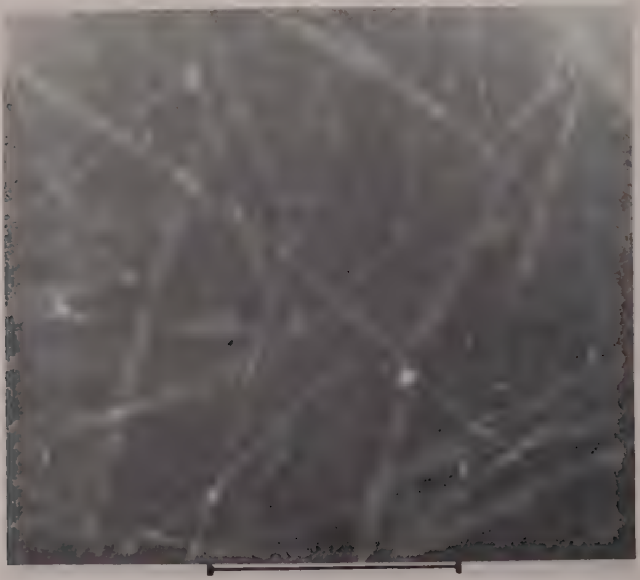

FIG. 5.

A micrograph of a dilute suspension of the tobacco mosaic virus protein.

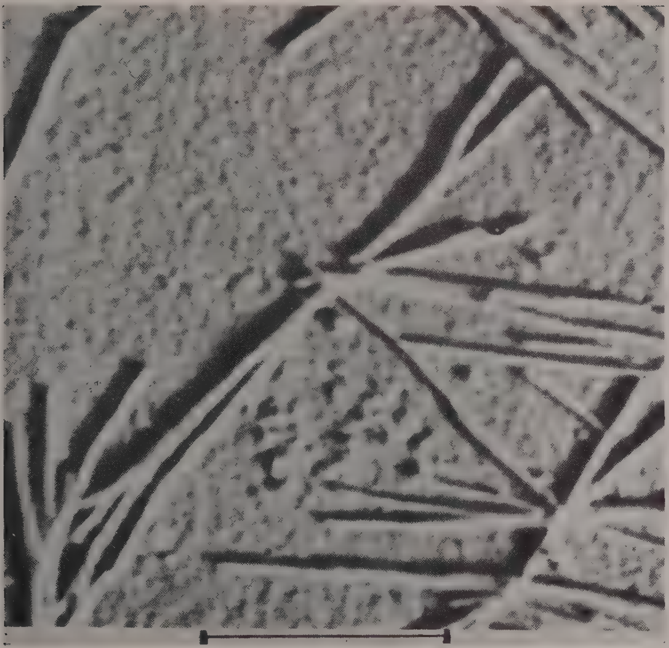

FIG. 6.
A field of a similar but more dilute tobacco mosaic protein suspension after shadowing.

or sunset, which would be invisible to him at noon, so metal shadow-casting brings out much detail that is not to be seen in an unshadowed (or in a vertically coated) preparation.

The PR-8 influenza virus used in this work has been purified by the differential high speed centrifugation of allantoic fluid of diseased chick embryos. Our centrifugal cycle did not differ in any significant detail from that employed by others[3] for the purification of this virus; following their suggestions it has been found advantageous to suspend the sedimented virus pellets in $CaCl_2$-Ringer solution and to make further necessary dilutions with 0.02M $CaCl_2$ solution. The tobacco mosaic virus was an ultracentrifugally purified preparation kindly furnished by W. M. Stanley. Dilutions of this virus have been made in distilled water. Both viruses have been prepared for microscopy by placing suitably diluted micro-drops on the usual collodion-covered screens and withdrawing as much as possible of the droplets after they have stood in contact with the screens for from 2 to 5 minutes. When a photograph of the same electron microscopic field was wanted before and after metal-shadowing the evaporated metal film was laid down without removing the preparation from its holder.

The electron microscope was a Type EMB instrument manufactured by RCA. The originals of the photographs of this paper were taken at 10000x on fine grained plates. Magnification was established with a thin metallic replica of a 15000 line-per-inch grating.

The shadowing metallic film was evaporated on to the specimens following the technique customary in this work. It is of course essential to use a metal that exhibits little structure of its own. Very few metals fulfill this requirement. Of those that do, chromium is satisfactory in a calculated thickness of ca 7 mμ

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[1] Jakus, M. A., Hall, C. E., and Schmitt, F. O., *J.A.C.S.*, 1944, **66**, 313.

[2] Williams, R. C., and Wyckoff, R. W. G., *J. Applied Physics*, 1944, **15**, 712.

[3] Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunology*, 1943, **47**, 261.

and as such has been used throughout the present work. The best angle at which to deposit the metal film will depend on the preparation, being greater for the smaller objects. For our studies this angle has been such as to make the lengths of the shadows from 5 to 10 times the heights of the shadowed objects.

Fig. 1 is a micrograph of a typical PR-8 influenza virus preparation while Figs. 2 and 3 are similar fields after the deposition of chromium shadows. They and the other pictures are negatives; that is, areas that are black, including the shadows, are those that are relatively transparent to the electron beam. The numerous circular bodies in Fig. 1 show up in the shadowed figures as little globules; they are the same as the electron microscopic objects of ca 75 mμ diameter that have previously been identified with the elementary bodies of influenza. Shadowing makes it clear that many of the apparently elongated and giant forms seen in direct electron micrographs are clusters of these globular bodies. In the shadow pictures these chains and aggregates notably resemble microcolonies of streptococci and staphylococci[4] but as far as the pictures reproduced here are concerned no conclusions can be drawn from these resemblances.

Many particles much smaller than the influenza bodies are present in some fields of the shadowed preparations (Fig. 3). They probably are the same as the small particles previously described.[5] In addition to this particulate matter, shadowing also outlines any soluble material that may still be accompanying or contaminating the preparation. A small portion of such a mass with embedded influenza bodies is just on the edge at the top of Fig. 3. Similar masses in a variety of forms are common on other photographs we have taken.

To interpret these micrographs it is necessary to know how much of their fine detail is contributed by the structure of the collodion substrate and the metal film. Experience shows that the substrate is never entirely smooth and that its inequalities become more apparent as the shadowing is more oblique. The surface appearance of collodion films made in the same way and shadowed at the same angle is remarkably constant, but it is well to prepare blanks from each lot of collodionized screens. A field of such a control shadowed at a 5 to 1 angle and chosen to contain some dirt particles to aid in focusing is given in Fig. 4. The seemingly rougher texture of the collodion under more oblique shadowing is evident in the tobacco mosaic picture of Fig. 6.

As was shown several years ago[6] a purified preparation of the tobacco mosaic protein contains fine fibrils that differ widely in length but have a more or less constant diameter of ca 15 mμ. A direct electron micrograph of the protein used in the present experiments is reproduced in Fig. 5. After being shadowed by a layer of chromium deposited to give ten-to-one shadows, a field of this preparation containing only a few fibrils looks like Fig. 6.

The technique of shadow-micrography is of such evident help in determining the shape and size of these small objects that we are applying it to a more thorough study of influenza and other viruses, and of some of the larger macromolecules.

Summary. A new procedure is described for the electron microscopy of small objects. This technique, illustrated by photographs of purified influenza and tobacco mosaic viruses, involves the oblique evaporation of a thin film of metal over the preparation before micrography. The three-dimensional effect it produces gives new information about the heights and shapes of objects seen in the preparation.

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[4] Wyckoff, R. W. G., *J. Exp. Med.*, 1934, **59**, 381.

[5] Chambers, L. A., Henle, W., Lauffer, M. A., and Anderson, T. F., *J. Exp. Med.*, 1943, **77**, 265; Taylor *et al.* op. cit.

[6] Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, 1941, **139**, 325.

14919

The Significance of Changes in Adrenal Size After Periods of Anoxia in Rats.*

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It has been shown that enlargement of the adrenals occurs after periods of exposure to decreased atmospheric pressure.[1-4] Lawless[5] demonstrated an increase in the water content of the adrenals of rats exposed to a pressure of 254 mm Hg (28,000 ft) for 3½ hr. He suggested that the increased water might be associated with augmented adrenal activity.

In this laboratory, histological examination of the adrenals of rats sacrificed after decompression to 225 mm Hg (30,000 ft) for periods up to 24 hr revealed hyperemia and congestion concomitant with an increase in size. The glands of those animals surviving the longer periods of decompression at this pressure also showed necrosis, cellular atrophy and edema. It was decided, therefore, to determine whether the weight gain of the adrenals might be due to the increased blood content.

Male albino rats were weighed and placed in vacuum desiccators which were evacuated by water aspirators. The animals were brought to an equivalent altitude of 30,000 feet at the rate of 1500 ft per min. The pressure was maintained within 2 mm by a mercury valve. Food and water were withheld during the experiment. At the close of the decompression period, the animals were brought to atmospheric pressure at the rate of 3000 ft per min, weighed, and sacrificed by a blow on the head. The adrenals were immediately removed, freed from adhering tissues, weighed, and dried to constant weight at 100° C. Blood samples (2 cc) were taken, weighed and dried to constant weight. Control animals were treated in the same manner except that they were not decompressed. The rats were weighed to the nearest gram and the adrenals to the nearest tenth of a milligram. The data were analyzed statistically by means of the "t" test for significance as described by Snedecor.[6] In all of the analyses there were 18 degrees of freedom, hence the t values must be greater than 2.1 or 2.88 to be significant at the 5 or 1% levels respectively.

The results are expressed in the accompanying table. Starvation alone, for the periods in these experiments, produced no effect; therefore, one set of figures, that of the fed controls, is used as a basis of comparison with the experimental values. The water content of the blood, which was 79.6%, did not change.

At the end of 7 hr exposure to anoxia there is an average increase of 0.0028 g in the absolute weight of the adrenals. When this is expressed as grams of adrenal tissue per 100 g of body weight, the increase which is 0.0060 g is statistically significant, t being 4.55. The weight of water contained in the glands has also significantly increased by an average of 0.0024 g, t being 15.77. The percentage of water in the glands has increased 1.9%.

The adrenals of animals exposed to anoxia for 24 hr show a decrease of 0.0029 g in weight. Despite this loss in absolute weight there is a slight gain of 0.0002 g per 100 g of body weight. This increase is statistically insignificant, t being 0.18. The actual weight of water has decreased but the percentage of

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* Aided by a grant from the National Research Council Committee on Research in Endocrinology.

[1] Earlier work reviewed by Tepperman, J., Engel, F. L., and Long, C. N. H., *Endocrinology*, 1943, **32**, 373.

[2] Sundstroem, E. S., and Michaels, G., *Memoirs of the Univ. of Calif.*, 1942, **12**, Univ. of Calif. Press, Berkeley.

[3] Darrow, D. C., and Sarason, E. L., *J. Clin. Invest.*, 1944, **23**, 11.

[4] Tornetta, F. J., Gordon, A. S., D'Angelo, S. A., and Charipper, H. A., *Fed. Proc. Amer. Soc. Exp. Biol.*, 1943, **2**, 50.

[5] Lawless, unpublished work quoted in Van Liere, E. J., "Anoxia," 1942, p. 206, U. of Chicago Press, Chicago, Ill.

[6] Snedecor, G. W., *Statistical Methods*, 1940, The Iowa State College Press, Ames, Iowa.

TABLE I.
Effect of Anoxia on Adrenals.
(Avg. and range: 10 rats on each point.)

		Body wt g	Adrenal wt g	Adrenal wt g per 100 g body wt	Water wt g	Water content %
Controls	Fed	176.6	0.0271	0.0159	0.0181	66.9
		(131-228)	(0.017-0.039)	(0.010-0.022)	(0.011-0.026)	(63.9-72.0)
	Starved 7 hr	161.9	0.0270	0.0168	0.0180	66.9
		(149-200)	(0.020-0.035)	(0.010-0.020)	(0.014-0.023)	(63.5-69.0)
	" 24 "	159.1	0.0265	0.0167	0.0177	66.8
		(147-174)	(0.024-0.029)	(0.014-0.018)	(0.016-0.020)	(64.3-68.4)
	Anoxic 7 "	140.6	0.0299	0.0219	0.0205	68.6
		(128-154)	(0.022-0.034)	(0.019-0.027)	(0.015-0.024)	(62.1-73.0)
	" 24 "	151.1	0.0242	0.0161	0.0166	68.6
		(135-175)	(0.022-0.028)	(0.015-0.019)	(0.015-0.020)	(58.3-78.5)

water is still elevated over that of the controls.

It may be seen, therefore, that at the end of 7 hr exposure to anoxia there is an increase in weight of the adrenals which amounts to 0.0060 g per 100 g of body tissue. The water in the adrenals of the control animals amounted to 0.0106 g per 100 g of body weight (0.0159 × 0.669). In the 7-hr group the water content has risen to 0.0150 g per 100 g of body weight, an increase of 0.0044 g per 100 g of body weight. If the increase in weight were due to an increased blood content, the figure 0.0044 would represent only 79.6% of the weight of the blood, as 20.4% would be solid matter which would not be lost by drying. This residue would, therefore, amount to 0.0011, thus accounting for 0.0055 of the increase of 0.0060 g per 100 g of body weight.

It is also evident that at the end of 24-hr exposure to anoxia there is an absolute loss of adrenal weight. This is probably due to a loss of tissue, since the water content has actually increased and histological examination reveals areas of necrosis, exhaustion atrophy and edema.

These results indicate that the immediate response of the adrenal to anoxia is hyperemia. It is suggested that, perhaps, this occurs as a result of other forms of stress. Observations on wet weights alone, made at an early stage would give a false impression of hypertrophy. The augmented blood supply is probably an indication of hyperactivity. Evidence of an increased demand for adrenal cortical factors has been offered by others[1-3] and by Dr. K. A. Brownell in unpublished observations on the effects of anoxia on carbohydrate metabolism made in this laboratory. Continued exposure to the decreased oxygen tension at 30,000 feet causes exhaustion atrophy of the cortical cells of the adrenal.

Acclimatization to an equivalent altitude of 30,000 ft has not been demonstrated. It is highly probable that, at altitudes at which acclimatization can occur, the adrenals would show hyperemia but would not undergo exhaustion atrophy. Instead, it is felt that recovery followed by true hypertrophy as evidenced by a gain in dry weight would occur. When the question of hypertrophy of the adrenal is being considered, it is important to determine wet and dry weights of the gland to ascertain whether the observed weight gain is due to hyperemia or is caused by an increase in solids. As lipids appear to play so dominant a role in adrenal cortical activity, it would be valuable to determine the changes in these substances.

The author wishes to express his thanks to Dr. Ralph A. Knouff for confirming the findings of the histological examinations.

Summary. Evidence is presented to show that the weight increase of the adrenals of rats exposed to a pressure of 225 mm Hg for 7 hr is due to hyperemia, indicating hyperactivity of the gland in response to the stress of anoxia. At this reduced pressure, the gland is unable to respond to the body's requirements and undergoes exhaustion atrophy as evidenced by histological examination and wet and dry weight determinations at the end of 24 hr decompression.

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